

Improved detection of *Clostridium difficile* and a new  
method to type highly lethal hospital-acquired  
infections due to *C. difficile*

A thesis submitted in fulfilment of the requirements for the degree  
of Master of Applied Science (Applied Biology and  
Biotechnology)

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## DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged.

A handwritten signature in blue ink, appearing to read 'Mohamed Said', is written over a horizontal line.

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*IN THE NAME OF ALLAH THE MOST  
GRACIOUS THE MOST MERCIFUL*

*To my father and mother,*

*Moftah and Salma*

*To my wife*

*Nagia*

*To my children*

*Fatma Ezzahra, Rem, Abdulrhman and Abdulmohimen*

*To my brothers and sisters,*

*I dedicate this work*



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## ABBREVIATIONS

AAD	Antibiotic-associated diarrhoea
AP-PCR	arbitrary-primer PCR
CCCNA	cell culture cytotoxicity neutralization assay
CDAD	<i>Clostridium difficile</i> - associated disease
CDI	<i>Clostridium difficile</i> infection
CdtA and cdtB	<i>C. difficile</i> binary toxin
CTA	cytotoxicity assay
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
EIA	Enzyme immunoassays
HRM	High Resolution Melt analysis
IC	Internal Control
ISR	Intergenic spacer region
Min	Minute
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NAP1	North American (NA) pulsed-field type 1
PaLoc	Pathogenicity locus
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMC	Pseudomembranous colitis
REA	Restriction Endonuclease Analysis
RFLP	Restriction fragment length polymorphisms
ssDNA	single stranded DNA
<i>tcdA</i>	<i>C. difficile</i> enterotoxin A gene
<i>tcdB</i>	<i>C. difficile</i> cytotoxin B gene
UPGNA	Pearson coefficient with arithmetic mean

## ABSTRACT

The incidence of hypervirulent epidemic *Clostridium difficile* has increased around the world and now in Australia. Assays that are capable of rapidly identifying these strains would enable earlier diagnosis and timely infection control response. The aims of the first part of the study were to validate and develop a molecular technique for the rapid diagnosis of toxigenic *C. difficile* from faecal samples using a multiplex real-time PCR assay and the development of a real-time PCR assay to identify strains carrying the frame shift mutation in the *tcdC* gene characteristic of hypervirulent strains. Seventy stool samples were selected that had been tested for *C. difficile* cytotoxin by cytotoxicity assay (CTA) and had *C. difficile* cultured. The DNA was extracted from *C. difficile* isolates and stool samples and tested in a conventional PCR for toxin genes. DNA extracted from stool samples was tested in real-time PCR. A real-time PCR targeting the *tcdC* frameshift mutation at position 117 ( $\Delta$ 117PCR) was investigated for identifying ribotype 027 *C. difficile* directly from stool samples. Thirty-three of 70 stool samples were CTA positive and a multiplex real-time PCR assay agreed with 32 of these samples. Additional samples were confirmed positive by real-time PCR and conventional PCR of DNA from pure culture. The  $\Delta$ 117 PCR assay detected the *tcdC*  $\Delta$ 117 mutation in 1 of 70 samples and this isolate was confirmed as ribotype 027. Using CTA as the “gold standard”, the sensitivity and specificity for the multiplex real-time PCR were 97% and 51.4% respectively. Comparing conventional PCR results of toxin genes from isolate DNA, the sensitivity and specificity for the multiplex real-time PCR were 100% and 80% respectively, and comparing conventional PCR results of toxin genes from stool samples, the sensitivity and specificity for the multiplex real-time PCR were 100% and 71.4% respectively.

In the second part of this study we investigated a high resolution melt analysis (HRM) of PCR ribotyping products. DNA was extracted from 93 clinical isolates confirmed as *C.*



*difficile* through conventional tests and five control strains (ATCC 9689, R20291, CD196, KI and M7404 – the later four being well characterised ribotype 027 strains). Ribotyping was performed using the published primers of Bidet et al. (1999) and band patterns were analysed using GelCompar II. The same primers were used to perform real-time PCR. The PCR normalised melt curves were imported into *ScreenClust* software (QIAGEN) to generate principal component analysis graphs depicting clustered relationships of strains. Ribotyping produced clear PCR bands for 88/98 isolates tested. A dendrogram generated by GelCompar II showed a diversity of ribotype patterns amongst these 88 clinical isolates with 18 groups identified with 70% homology. Three of the four control 027 ribotype isolates showed 100% homology (R20291, KI and CD196). The fourth showed 82% homology with the other 027 control strains. One clinical isolate showed 98% homology with the control 027 strains and was shown to produce the toxins *tcdA*, *tcdB*, *cdtA* and *cdtB* and contained the frameshift mutation characteristic of epidemic 027 strains. The patient harbouring this strain died from complications of chronic disease and there was no evidence of spread of this strain to other patients. *ScreenClust* analysis of the same 88 HRM results showed clustering of isolates, with 027 strains identifiable as a unique cluster. HRM analysis correctly identified the control 027 stains and the clinical isolate shown also to be 027.

The real-time PCR assay of toxin genes in stool was performed in 4 hours and thus can serve as a rapid assay for patients suspected of having CDI. A real-time PCR targeting the *tcdC* frameshift mutation at position 117 successfully identified a ribotype 027 strain in our patient population. HRM analysis of the real-time PCR products of the intergenic (16S-23S rDNA) spacer region has enabled the identification of ribotype 027 hypervirulent strains. It has enabled the identification to occur within 2 – 3 hours of colony isolation and thus is a

valuable aid in the timely identification of these strains so that infection control can be rapidly implemented.

## **CHAPTER 1**

**A review of infection and diagnosis of *Clostridium difficile***

## 1. Introduction

*Clostridium difficile* is a well-established human pathogen causing a range of symptoms from mild to severe diarrhoea, and is the etiological agent of pseudomembranous colitis (PMC). Infection by this pathogen was found to be associated with the use of gastric acid suppressing agents, particularly proton pump inhibitors and antibiotics which allow survival of spores in the stomach and selection for *C. difficile* in the gut (Dial et al., 2005, Anand et al., 1994). However, *C. difficile* is not the main agent of mild antibiotic-associated diarrhoea in out-patients (Beaugerie et al., 2003).

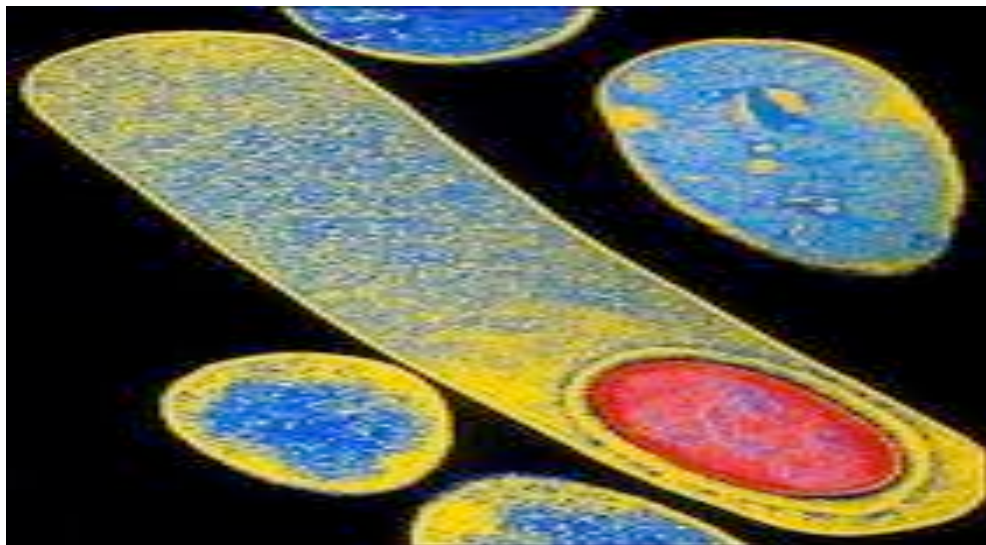
The first description of this organism was made by Hall and O' Toole in 1935 where the species name "*difficile*" came from the difficulty encountered during the first attempts to culture this organism. In addition, the organism requires at least 48 hours of uninterrupted anaerobic growth in order to see colonies (Hall and O'Toole, 1935). Wide distribution and use of antibiotics in Europe and North America after the Second World War is considered as the most important cause for the emergence of a new type of 'diphtheritic-like' PMC. The extensive use of some antibiotics such as clindamycin and lincomycin was clearly associated with this form of disease (Tedesco et al., 1974). In 1977 a previously uncharacterized cytopathic toxin from the stool of patients suffering from severe PMC was described. This toxin was suggested to be responsible in this case of inflammation, but the source and nature of this toxin was not pursued (Larson et al., 1977). Kuijper et al. (1987) reported that *C. difficile* was responsible for PMC as well as being responsible for some cases of antibiotic- associated diarrhoea (Kuijper et al., 1987).

*C. difficile* is the most frequently identified enteric pathogen in patients with antibiotic-associated diarrhoea and colitis (Bartlett, 1994). It accounts for 10%-25% of all cases of antibiotic-associated diarrhoea and virtually all cases of antibiotic-associated PMC

(Bartlett, 1994). Since the turn of the century the incidence of *C. difficile* infection has increased and major outbreaks in hospitals throughout many developed countries have been reported (Loo et al., 2005). The incidence of *C. difficile* infection has increased markedly in many countries and this increase has been suggested to be due to the emergence of a new hypervirulent epidemic strain known as North American PFGE type 1, REA group BI, or PCR ribotype 027 (Loo et al., 2005).

### 1.1 General description of *C. difficile*

*C. difficile* is a Gram-positive, anaerobic bacillus belonging to the family of *Clostridiaceae*. It has the ability to form subterminal endospores (Figure 1.1) in response to environmental stress (Voth and Ballard, 2005).



**Figure 1.1** Coloured transmission electron micrograph of *C. difficile* showing subterminal spore (Science Photos Library, [www. science photo.com](http://www.sciencephoto.com)).

This bacterium is widely distributed in soil and contaminates environments when a host excretes the organism in its feces (al Saif and Brazier, 1996).

## **1.2 Antibiotic-associated diarrhoea (association with human and animal disease)**

Antibiotic-associated diarrhoea (AAD) is defined as a case of diarrhoea that happens after administration of antibiotics with the frequency and severity of this complication differing depending on the type of antibacterial agent (Bartlett, 2002). Clindamycin, cephalosporins, broad-spectrum penicillins, and fluoroquinolones are most implicated with the problem though all antibiotics have been implicated (Bignardi, 1998).

The incubation period before symptoms appear is variable. Symptoms can appear after one day of exposure to antibiotics, but it also has been reported that symptoms can arise six weeks after the first dose of antibiotic (Anand et al., 1994, Kelly et al., 1994). “The rates of diarrhoea associated with parenterally administered antibiotics, especially those with enterohepatic circulation, are similar to rates associated with orally administered agents” (Wistrom et al., 2001). Table 1-1 lists the variety of antibiotics and their risk level as a predisposing factor for *Clostridium difficile*- associated disease (CDAD) (McFarland et al., 2000). Recently, fluoroquinolones have been reported along with cephalosporins as a major predisposing factor in the development of CDAD (De Andres et al., 2004).

The incidence of AAD in adult hospitalised patients has been reported at 13-29%, and may rise to 60% during hospital outbreaks, but was less than 0.1% in out-patients (Levy et al., 2000, McFarland, 2008). It has been proposed that the primary cause of AAD is the disruption of intestinal normal flora by antibiotics leading to overgrowth and colonisation of pathogens in the intestine. The most commonly diagnosed and potentially severe form of AAD is caused by *C. difficile*. This pathogen is implicated in 20% to 30% of patients with AAD, in 50% to 70% of those with antibiotic-associated colitis, and in nearly all cases of antibiotic-associated PMC (McFarland, 1998, McFarland et al., 1999). Besides *C. difficile* infection, other factors involved in AAD include overgrowth of other pathogens, impaired

faecal fermentation, and changes in dietary fibre intake (Clausen et al., 1991, Oldfield, 2004).

**Table 1.1** Antibiotics associated with the development of CDAD.

<b>High Risk</b>	<b>Moderate Risk</b>	<b>Low Risk</b>
Clindamycin	Quinolones	Aminoglycosides
Ampicillin and Amoxicillin	Tetracycline	Bacitracin
Lincomycin	Sulfonamides	Metronidazole
Cephalosporins (2nd and 3 <sup>rd</sup> generation)	Erythromycin	Vancomycin
	Chloramphenicol	
	Trimethoprim	

Reproduced from McFarland et al. (2000).

### 1.3 Spread and surveillance of *C. difficile*

Contaminated hands of healthcare staff and the people who come in contact with infected patients, in addition to environmental surfaces (e.g. floors, bedpans, toilets) contaminated with the bacteria or its spores play an important role in the spread of this pathogen throughout hospitals. This pathogen can produce spores when in unfavourable environmental circumstances such as being outside the body. These spores remain viable for a long period of time on clothes and environmental surfaces and can germinate when there is a return to favourable environmental conditions.

In Canada and the United States outbreaks of severe *C. difficile* infection (CDI) have increased markedly since 2003 (McDonald et al., 2006, Warny et al., 2005). The emergence and rapid spread of a specific strain of *C. difficile* was the main reason for these outbreaks. This strain is characterized by the production of high rates of cytotoxins and enterotoxins *in vitro* and with the presence of a third toxin named binary toxin (Warny et al., 2005, Barbut

et al., 2005). In northern Europe (United Kingdom (UK), Belgium and the Netherlands) the epidemic strain began to spread in 2003 (Smith, 2005, van Steenberghe et al., 2005). Recently, this strain has been detected in Western Australia (Riley et al., 2009), Victoria (Richards et al., 2011) and Sydney (Huntington et al., 2011).

A proposed surveillance system for *C. difficile* infections was published by McDonald et al. and has been adopted in Europe and the United States (McDonald et al., 2007, Cohen et al., 2010). In 2010 the Australian Commission for Safety and Quality in Healthcare has gained jurisdictional agreement with Australian states to implement surveillance for CDI using an approach consistent with that proposed by McDonald et al. (2007) (Australian Commission for Safety and Quality in Healthcare, 2010).

Presently, the nationwide rates of *C. difficile* are unknown across Australia due to inconsistencies in the way surveillance data is collected. Rates between 1.27 and 2.3/10,000 bed days have been reported in some states (McGregor et al., 2005, McGregor et al., 2008). These reported rates are in contrast to rates of between 3.8 and 9.5/10,000 bed days reported in Canada in 1997 and 2005 (Cohen et al., 2010). With the appearance of the epidemic strain in Quebec, Canada, the rates of CDI have increased four times in Quebec (Pepin et al., 2005). It has been suggested that the implementation of CDI surveillance programs supported by international recommendations would play an important role in the reduction of infection rates (Cohen et al., 2010, McGregor et al., 2008).



## 1.4 Pathogenesis

### 1.4.1 Initiation of disease

*C. difficile* will often lie dormant as a spore waiting for an opportunity to germinate and begin to multiply. If a patient has received antibiotics, much of the normal bowel microbiota can be wiped out and hence *C. difficile* will germinate and grow into large numbers. As *C. difficile* grows it may produce a potent cytotoxin that can kill intestinal cells and also an enterotoxin that cause water to leave the remaining cells resulting in diarrhoea (Kelly et al., 1994). In animals *C. difficile* infection has been known as a cause of colitis (Belanger et al., 2003). In humans this organism was not known as a cause of diseases until 1978, when the disease was described and named as pseudomembranous colitis (Figure 1.2). This condition can be complicated by more serious diseases such as toxic megacolon, perforation and peritonitis (Poxton et al., 2001).



**Figure 1.2** PMC, post-mortem specimen. Note the areas of intense inflammation (Poxton et al., 2001).

The mode of disease transmission of *C. difficile* is through the faecal oral route. Ouwehand et al. (2002) have stated the digestion and absorption of food and the generation of antibodies is the key role of intestinal microflora (Ouwehand et al., 2002). The size of the pathogen population is controlled by being prevented from overgrowth by the

microflora which form part of the intestinal mucosal barrier; this barrier is regulated by the immune system (Poutanen and Simor, 2004, Wilson, 1993).

The mechanism of *C. difficile* infection and pathogenesis was first demonstrated by the use of a hamster model (Wilson, 1993). When vegetative cells and spores of *C. difficile* are ingested, most of the vegetative cells are killed by stomach acid and only about 1% reaches the small intestine. These vegetative cells are inhibited by intestinal microflora and are removed from the body in the stool. Spores however can survive in the stomach, pass through to the small intestine and the action of bile salts induces spore germination. In the absence of microflora these germinated cells can colonise the colon and toxigenic strains will produce two large toxins named toxin A and toxin B. "Both toxins disrupt the actin cytoskeleton of intestinal epithelial cells by uridine diphosphate-glucose dependent glycosylation of Rho and Ras proteins" (Rupnik et al., 2005). It has also been suggested that these toxins bind to the receptors on epithelial cells resulting in an inflammation followed by diarrhoea. These toxins then lead to breakdown of the junction between epithelial cells which is then followed by perforation and penetration in the intestine (Starr, 2005). It has been reported that toxins A and B stimulate the release of tumor necrosis factor-alpha and proinflammatory interleukin, which produces pseudomembrane formation and the inflammatory process (Poxton et al., 2001).

#### **1.4.2 *C. difficile* toxins (A and B)**

It was noted that a culture filtrate of this organism could kill inoculated rabbits, even though the organism was first isolated from healthy infants (Hall and O'Toole, 1935). When CDI was first investigated, the two toxins were described independently and initially confused (Larson et al., 1977).

A large toxin was found when ion exchange chromatography was used to separate secreted proteins as reported by two independent research groups in 1981, that toxin was named as toxin A (Taylor et al., 1981, Banno et al., 1984), toxin A was able to produce fluid accumulation in ligated ileal loops of rabbits while toxin B could not; in addition, toxin A produced less rounding of fibroblasts in culture. “Toxin A produced a similar profile as total supernatant, when given intragastrically, which toxin B did not, unless mixed with small amounts of toxin A, or given to animals with bruised ceca” (Lyerly et al., 1985).

We now know that disease-causing *C. difficile* produce at least two toxins (Lyerly et al., 1988). Experiments on laboratory animals showed that toxin A has a cytopathic effect on cells while toxin B caused epithelial cell rounding and death and is up to a thousand-fold more toxic in laboratory cell culture assays (Voth and Ballard, 2005). Toxin A was thus thought to be the principal virulence factor while toxin B required toxin A to be present and begin the cascade of break to the colonic mucosa and create disease (Lyerly et al., 1988).

Before the development of genetic tools to manipulate *C. difficile*, natural mutations and variations in the toxin genes of *C. difficile* were sought out to shed some light on their function and role in pathogenesis, especially in the debate as to which toxin was most important. Some of *C. difficile* strains were clinically reported to cause disease when they produced no detectable toxin A. This challenged the current thinking, and was not only academically interesting, but it also had ramifications for clinical practice. At that time, clinical immunoassays only tested for the presence of toxin A.

Sequencing of the reference strain VPI 10463 has revealed that there are five genes within a 19.6 kb pathogenicity locus (PaLoc) known as *tcdA*, *-B*, *-C*, *-E* and *-R* (Figure 1.3) (Hammond and Johnson, 1995). The two major toxins encoded by the pathogenicity locus

are toxin A (primarily an enterotoxin) and toxin B (a cytotoxin). Strains that are non-toxigenic have a 115-bp DNA fragment bordered by two insertion sequences which has replaced PaLoc (Braun et al., 1996, Cohen et al., 2000, Hammond et al., 1997).

Strains reported to be toxin A negative and toxin B positive have been isolated from patients with CDAD and colitis (Savidge et al., 2003). Strain 8864 was the first toxin A-negative toxin B positive (A-/B+) described (Lyerly et al., 1992), producing no toxin A *in vitro* by immunoassay, and a toxin B found to be more lethal and weakly enterotoxic to animals. Sequencing of the TcdA gene and TcdC gene of strain 8864 was later shown to have deletions at the 3'ends of these genes. This truncation in the resulting toxin A protein lacks the receptor binding repeats and translocation domain (Soehn et al., 1998). While isolates like strain 8864 remain rare, other disease-causing A-B+ strains have been reported more frequently in the early 1990's. Strain 1470 and related isolates (Depitre et al., 1993, Sambol et al., 2000), also have deletions in *tcdA* and are negative in clinical tests for toxin A. They were initially thought to be non-pathogenic in mice (Depitre et al., 1993), but further work in the more sensitive hamster model (Sambol et al., 2001) and fatal cases in humans (Johnson et al., 2001) and human epidemics soon dismissed this idea (Alfa et al., 2000, Kuijper et al., 2001). Toxin B from these strains was found to share homology with a toxin from a related *Clostridium* species, TcsL from *C. sordelii* (Chaves-Olarte et al., 1999), in its enzymatic domain, which allows it a larger range of host molecule specificity and increased toxicity. This variation in Toxin B's enzymatic domain left the debate concerning toxin A versus B open.

Stabler et al. (2008) have reported the results of assays that showed the toxin B has more binding capacity than toxin A. Both toxin A and B have a cytotoxic effect on many different cell types. Others have reported that the effect of toxin B is greater than the effect

of toxin A and both lead to increased vascular permeability due to breakdown of the junction between epithelial cells that can result in haemorrhage (Borriello, 1998, Poxton et al., 2001).

Recent advances in the genetic manipulation of *C. difficile* have provided a molecular scientific answer to the question of which toxin is most important to disease. Using an unstable plasmid as a recombination vector produced isogenic mutants of *tcdA* and *tcdB* in strain 630 (Lyras et al., 2009, O'Connor et al., 2006). When these mutant strains were introduced into the hamster model, it was shown that toxin B was the required virulence factor. Hamsters infected with the toxin A mutants showed no difference in mortality as compared to the wild-type. In contrast, the group of hamsters infected with the toxin B mutant *C. difficile* showed significantly less mortality, and analysis of the *C. difficile* obtained from the fatal cases indicated that they were revertant strains in which the recombination vector was excised: this supported the idea that toxin B production is favoured for pathogenesis and survival in the gut (Lyras et al., 2009).

However, a more recent study has reported conflicting results. Using the ClosTron system of a re-targetable group II intron (Heap et al., 2007), null mutations were made in *tcdA* and *tcdB* (Kuehne et al., 2010). In contrast to the previous study, both toxins contributed to virulence in the hamster model. However, hamsters infected with *C. difficile* lacking toxin B survived for several days longer than those infected with *C. difficile* lacking toxin A (Kuehne et al., 2010). While both may participate in the disease process, it appears that toxin B makes the larger contribution to virulence.

### **1.4.3 Binary toxin (*cdtA* and *cdtB*)**

Several gram-positive spore-forming species produce another type of toxin called binary toxin. This toxin has been particularly well characterized among *Clostridium* and *Bacillus* spp. (Barth et al., 2004) and is produced in a minority of strains from patients diagnosed with PMC and is considered as one of the characteristics of the current hypervirulent strain (Cloud and Kelly, 2007).

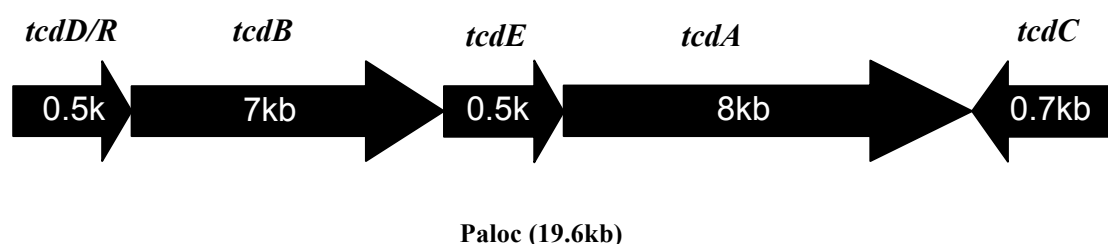
Binary toxin is not found in historical strains, but is produced by strains isolated from pigs and calves (Keel et al., 2007). Binary toxin is composed of two unlinked components, the enzymatic and the binding components which act as an actin specific ADP ribosyltransferase (Perelle et al., 1997). Barbut et al. (2005) reported that the binary toxin genes (*cdtA*) and (*cdtB*) together encode this toxin and induce the production of an actin specific ADP- ribosyltransferase which leads to a breakdown of the actin skeleton, followed by cytopathic effects in cell lines (Barbut et al., 2005).

### **1.4.4 Putative negative regulator (TcdC)**

The TcdC gene is considered as a negative regulator gene for both toxins A and B (Spigaglia and Mastrantonio, 2002). The hypervirulent strain recently involved in epidemic (PCR ribotype 027 in Europe and North American (NA) pulsed-field type 1 (NAP1/ BI) in the USA) is said to cause more severe disease and lead to higher rates of morbidity and mortality than other strains of *C. difficile* (Kuijper et al., 2006). The severity of the hypervirulent strain is thought to be due to the production of high levels of TcdA and TcdB toxins caused by two deletions detected in this *tcdC* gene (Warny et al., 2005).

Sequencing of ribotype 027 strains revealed an 18 bp deletion in the TcdC toxin repressor gene, in addition to a highly-conserved point deletion at position 117 ( $\Delta 117$ ) in *tcdC* that

introduces a frameshift mutation truncating the predicted TcdC product to 65 amino acid residues (MacCannell et al., 2006). Thus the presence of  $\Delta 117$  mutation in the TcdC gene can be used as a marker for the detection of the 027/NAP1/ BI epidemic strain (MacCannell et al., 2006). In recent years many studies using real-time PCR assays targeting the  $\Delta 117$  mutation in *tcdC* for detection of the epidemic strain have been conducted including melting curve analysis (Sloan et al., 2008, Wolff et al., 2009).



**Figure 1.3** Pathogenicity locus of *C. difficile* and the domain distribution of TcdA, TcdB and TcdC genes.

## 1.5 Laboratory diagnosis of *C. difficile*

### 1.5.1 Culture

In order to identify disease producing strains of *C. difficile*, laboratories use different types of techniques including culture, cytotoxicity assay (CTA), or enzyme immunoassays (EIA) for the detection of toxigenic *C. difficile* in faecal samples (Turgeon et al., 2003). Detection of toxigenic *C. difficile* by isolation of pure culture is the most sensitive laboratory test in clinical use, but it is not as specific as the cell cytotoxicity assay and is time consuming (Gerding et al., 1995). Weese et al. (1999) reported that the lack of specificity in determining toxin producing strains may result in the reporting of toxin-negative strains and the delay in sample processing may lead to a reduction in the survival rate of organisms in stool samples resulting in false negative culture results (Weese et al., 1999).

There is still much debate about patients who were diagnosed with CDAD and have a negative stool toxin test, but have *C. difficile* recovered from their stool specimens by

culture. Because nontoxigenic strains are not considered pathogenic, the determination of *in vitro* toxin production by isolates cultured from toxin negative stools may help somewhat to resolve this dilemma (Shanholtzer et al., 1992).

### **1.5.2 Toxin detection**

Toxin B in stool can be used as a marker for the presence of toxigenic bacteria in a stool sample (Gerding et al., 1995). The cytotoxicity assay was considered as the gold standard for detection of *C. difficile* toxin B in stool, where the sensitivity can be  $\leq 85\%$  (Bouza et al., 2001). This percentage rises to  $\leq 99\%$  when the cytotoxicity assay is combined with toxigenic culture (i.e., *C. difficile* broth culture followed by CTA performed on the culture broth); however this improvement in sensitivity is associated with an increased cost of detection (Bouza et al., 2001, Peterson et al., 1996). In addition, the final reading of this test can take up to 4 days of incubation and requires tissue culture facilities (Peterson et al., 1996).

Many researches state that the detection of *C. difficile* by toxigenic culture is more sensitive than the cytotoxicity assay, even though the specificity is still under review (Bartlett and Gerding, 2008).

Enzyme immunoassays (EIA) based on monoclonal antibodies against toxin A and/or B have been commonly used for toxin detection, although these EIAs are not as sensitive as the cytotoxicity assay and the specificity of such assays have been questioned (Barbut et al., 1993, Shanholtzer et al., 1992).



### 1.5.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is used for the detection of toxin genes of *C. difficile*. PCR methods are more rapid and have higher sensitivity and specificity when compared to culture-based methods (Louie et al., 2000). False positive PCR results obtained by amplification of nonspecific products have been decreased by the use of fluorescent labelled probes in real-time PCR assays (Houser et al., 2010). This is because binding of the probe as well as the primers to the target sequence is needed for a signal to be produced. The accuracy of PCR detection methods has become more reliable due to the specificity of primers and probes to DNA targets (Louie et al., 2000).

A traditional PCR assay targeting the *tcdB* gene only has been developed where the specificity and sensitivity were 100% and 91.5% respectively (Guilbault et al., 2002). Van den Berg et al. reported similar results when cytotoxicity assay results were compared to the results of a real-time PCR assay targeting the *tcdB* gene. The sensitivity, specificity, positive predictive value, and negative predictive value were 100%, 97%, 55%, and 100%, respectively when a cytotoxicity assay was used as gold standard (van den Berg et al., 2006). In a study presented in 2007, it was reported that culture concordance values of VIDAS immunoassay, the PTAB immunoassay, and a real-time PCR assay were 53.6%, 55.4%, and 71.4%, respectively when compared with the cell cytotoxicity assay (van den Berg et al., 2007). The sensitivity, specificity, positive predictive value and negative predictive value of PCR assay targeting *tcdB* was 93.3%, 97.4%, 75.7%, and 99.4%, respectively when compared with enzyme immunoassay (Peterson et al., 2007). A comparison of a commercial real-time PCR assay for *tcdB* detection and cell cytotoxicity assay to toxigenic culture which is considered as the gold standard showed a sensitivity, specificity, positive predictive values, and negative predictive values of 83.6%, 98.2%,

89.5%, and 97.1% and 67.2%, 99.1%, 93.2%, and 94.4 %, for real-time PCR assay and cell cytotoxicity assay, respectively (Stamper et al., 2009a).

In a comparison study between nine commercially available *C. difficile* toxin detection assays, Eastwood et al. reported that the real-time PCR assay had the highest sensitivity and highest negative predictive value than all other detection assays (Eastwood et al., 2009). Kvach et al. (2010) also reported that a real-time PCR assay was more sensitive ( $p=0.0001$ ) than a *C. difficile* toxin immunoassay. A multiplex assay targeting toxin genes *tcdA* and *tcdB* as well as *tpi* a housekeeping gene specific to *C. difficile* was developed by Lemee et al. (2004). It allows combined diagnosis and toxigenic type characterization for human and animal *C. difficile* intestinal infections (Lemee et al., 2004).

The emergence of new toxigenic *C. difficile* strains such as TcdA negative/TcdB positive strains as well as TcdA negative/TcdB negative/CDT positive strains make tests based on the detection of a single toxin unreliable (Geric et al., 2003, Drudy et al., 2007). Persson et al. (2008) developed a molecular method for the detection of pathogenic *C. difficile* isolates, including a multiplex PCR for detecting the genes encoding TcdA, TcdB and binary toxin; and a sequencing-based method for investigating deletions and premature stop codons in *tcdC* gene.

Recently researchers have reported some TcdA negative/TcdB negative strains shown to produce both *cdtA* and *cdtB*, suggesting binary toxin may be considered as a virulence factor since the binary toxin protein has the ability to increase the adherence ability of *C. difficile* to the intestinal wall (Schwan et al., 2009). In a study conducted by Geric et al. (2003) that reported the frequency of binary toxin genes (*cdtB* and *cdtA*) among *C. difficile* isolates that do not produce toxin A and B, it was found that 15.5% of *tcdA* and *tcdB* negative *C. difficile* isolates examined were positive for both *cdtA* and *cdtB*. Therefore,

assays that detect toxigenic *C. difficile* based on the presence of only *tcdA* and *tcdB* may miss isolates only carrying binary toxin. Until more is known about the significance of these isolates there is a need for a reliable, high throughput, multiplex real-time PCR assay capable of detecting genes encoding for all three toxins.

### **1.6 Typing of *C. difficile***

Typing of *C. difficile* can lead to a greater understanding of the strain causing disease in humans and the epidemiology of *C. difficile*. By typing different strains, we can track the spread of new strains throughout healthcare facilities and geographical areas and determine when outbreaks are occurring in a region. Although there are a variety of typing methods available for bacteria, not all typing methods are equally valuable for each species. Optimal typing methods also depend on the specific objectives and different typing methods may be better for different applications. In general, the main characteristic of a valuable typing methodology are discriminatory power, reproducibility, ability to standardise protocols and technical aspects such as ease and cost. By having a highly discriminatory typing method, strains that appear to be identical using one method can be differentiated using a different typing method. A highly discriminatory typing method is particularly useful when investigating local outbreaks. Reproducibility involves the ability to obtain the same results when the method is used at different times and by different laboratories. This is important for any comparisons that take place over time or where comparisons with external laboratories are needed.

There are many different typing methods proven to be effective in the typing of *C. difficile*. Some of these methods depend on phenotype such as lysotyping and others on genotype such as restriction endonuclease analysis (REA) of the total bacterial genome (Kuijper et al., 1987, Kato et al., 1993), pulsed-field gel electrophoresis (PFGE) of enzyme-restricted

whole genome DNA, arbitrarily primed PCR (AP-PCR) (Killgore and Kato, 1994, Fawley and Wilcox, 2002, Rodriguez-Palacios et al., 2007), and PCR-ribotyping (Gurtler, 1993).

Genotypic methods are based on the analysis of the genetic profile of the organism, whereas the analysis of gene expression is the main basis of phenotypic methods. Before plasmid and whole-genome restriction analysis become available as typing methods in the 1980's, serotyping was the most used phenotypic technique (Clabots et al., 1988, Kuijper et al., 2009). Due to the ease and rapidity of analysing DNA, whether amplified or not, genetic methods tend to be the most common methods used in recent years.

### **1.6.1 Pulsed-field gel electrophoresis (PFGE)**

Pulsed-field gel electrophoresis is an example of a typing method that depends on a whole-genome restriction analysis. After embedding cells in agarose the total genomic DNA is extracted using proteinase K and mild detergent. DNA fragments are produced after the whole genome is digested by restriction enzymes, these fragments are separated in a gel depending on its molecular weight in a specialized electrophoresis unit with a pulsing electrical field (Schwartz and Cantor, 1984).

Grouping and classification of isolates occur based on the bands produced by this method. PFGE was first applied to characterise *C. difficile* isolates by Kato and colleagues in 1994 (Kato et al., 1994). PFGE is considered the gold standard and characterized by very high discriminatory power for molecular typing of *C. difficile* (Kato et al., 1994). However, this method is not practical in an outbreak situation due to the length of the protocol as from 4 to 6 days is required to obtain typing results using this method. In addition, manual labour requirements, expensive equipment requirements, and hazardous chemicals used by this method have resulted in a decrease in the use of PFGE as a main typing method (Gal et al., 2005).

### **1.6.2 Restriction endonuclease analysis**

REA is another method using genomic-level comparison of restriction fragment length polymorphisms (RFLP) to differentiate between bacterial species or strains. It differs from PFGE, where rare cutting endonucleases are selected, and fragment sizes often range into the hundreds of kilobases while PFGE is able to resolve extremely large nucleic fragments. REA uses digestion of the whole genomic DNA by restriction enzymes resulting in fragments that are separated by electrophoresis (Clabots et al., 1993). The technique has a high discriminatory power, however it is technically demanding, the interpretation is subjective and data exchange between laboratories is difficult (Kuijper et al., 2009).

### **1.6.3 PCR-ribotyping**

PCR-ribotyping has been reported to provide a discriminatory, reproducible, and a simple alternative to other typing methods (Cartwright et al., 1995). PCR-ribotyping was first described by Kostman et al. in 1992 in a study of the epidemiology of *Burkholderia cepacia*, where it was proposed as an alternative to the more classical ribotyping methods (Kostman et al., 1992). Unlike traditional ribotyping, which analyses strain-specific differences in the coding sequences of the 16S and/or 23S ribosomal genes, PCR-ribotyping amplifies the intergenic spacer region (ISR) between the two, using primers from the highly-conserved 16S and 23S termini.

Gurtler in 1993 described the first PCR-ribotyping for *C. difficile* and demonstrated that *C. difficile* isolates may contain up to 16 different alleles of the rRNA operon, with varying intergenic topologies (Gurtler, 1993). The PCR-ribotyping technique has a number of advantages over other methods specifically PCR-ribotyping has been shown to be more discriminatory than arbitrary-primed PCR (AP-PCR) (Collier et al., 1996), and is quicker

and simpler than PFGE. PCR-ribotyping also avoids the excessive endogenous nuclease activity of some *C. difficile* isolates that renders them untypeable by PFGE (Stubbs et al., 1999). Furthermore, the PCR-ribotyping technique is "considered to be highly discriminative, reproducible and can be performed relatively easily and rapidly" (Arroyo et al., 2005). Because of this, PCR-ribotyping is more amenable to rapid and high-volume strain typing, or epidemiologic projects where only limited amounts of the genomic template are available for study (MacCannell et al., 2006).

### **1.7 The emergence of a hypervirulent strain**

From 2003 evidence emerged that *C. difficile* was becoming more virulent and the disease more complicated and more resistant to standard treatment. In the USA, Canada and Europe one strain of *C. difficile* associated with this severe disease was reported as ribotype 027 in Europe and NAP1/ BI in the USA (Cookson, 2007). The first report of PCR ribotype 027 was in 1988 from the culture collection of M. Popoff (Paris, France). This isolate was from a 28-year-old woman suffering from severe PMC (Kuijper et al., 2006).

Warny et al. (2005) have reviewed the emergence of ribotype 027 in the hospitals of Montreal and Southern Quebec, Canada and noted the increased incidence of infection. They also reported that the rates of CDI had been elevated between 2003 and 2004 and reached 14,000 nosocomial cases with an incidence of more than 15 per 10,000 in-patients in January 2005. This was reported to be five times greater than the historical average (Warny et al., 2005).

In the UK two outbreaks of CDI occurred with one outbreak reaching a mortality of approximately 11% in the period between 2003 and 2005 at the Stoke-Mandeville Hospital. These outbreaks were characterised by an increase in mortality and resulted in the deaths of

38 patients in total for both outbreaks (Buckinghamshire Hospitals, 2006). It has been proposed that the increased severity of this hypervirulent strain is related to high production of toxins A and B caused by two deletions in the TcdC toxin regulator gene (Spigaglia and Mastrantonio, 2002). In the USA there have been more than 250,000 cases of CDAD diagnosed per year, which costs the health care system over \$1 billion (Wilkins and Lyster, 2003).

Before the emergence of this epidemic ribotype 027 strain, data describing the incidence and prevalence of the various ribotypes of *C. difficile* in Australia had not been reported. The incidence of *C. difficile* in Western Australia hospitals during the 1980s was high but it declined “from 2.09 cases per 1,000 discharges in 1998 to 0.87 cases per 1,000 discharges in 1999” and was assumed to correlate with a decreased use of broad-spectrum cephalosporins (Thomas et al., 2002).

The first reported isolation of ribotype 027 in Australia occurred at the end of October 2008 in Western Australia when a 43-year-old woman presented with diarrhoea and a permanent ileostomy. She was believed to be infected while travelling in the United States (Riley et al., 2009). This isolate was positive for *tcdA* and *tcdB* as well as both *cdtA* and *cdtB* binary toxin genes and showed resistance to many antibiotics especially fluoroquinolones. In addition, the sequencing of the TcdC gene (putative negative regulator) indicated that the strain contained an 18 bp deletion as well as a one bp deletion at position 117 which is the characteristic feature of the ribotype 027 epidemic strain (Riley et al., 2009).

An epidemic hypervirulent 027 ribotype strain was also recognised in a hospital in Melbourne, Australia in late January 2010 (Richards et al., 2011), when an 83-year-old Latvian man was admitted to hospital. This patient had undergone an aortic valve

replacement for aortic stenosis. After five days of surgery the patient developed watery diarrhoea and *C. difficile* was isolated from a stool sample. This isolate was considered to be the first report of a ribotype 027 acquired in Australia (Richards et al., 2011). It is likely that the 027 strain is now well established in Australia as shown by the number of isolates reported by Huntington et al. (2011).

It has been reported that the levels of toxins A and B produced by the new epidemic strain PCR ribotype 027 are 16 to 23 times higher than toxin A and toxin B produced by non-027 toxigenic strains (Warny et al., 2005). In another study, it was reported that the risk of developing severe CDAD caused by this hypervirulent strain is twice that of the CDAD caused by a non-PCR ribotype 027 strains (Hubert et al., 2007). However, Carter et al., (2011) found that *tcdC*-status is not correlated with toxin production in clinical isolates. Hence, these authors have raised a question about the accuracy of assigning gene function by studying non-isogenic strains, particularly in a highly heterogeneous species such as *C. difficile*.

National surveillance programs do not cover the entirety of the European continent and an estimation of the incidence of ribotype 027 incidence is not easy. Nine of a total of 16 European countries have reported outbreaks due to ribotype 027 epidemic strain (UK, Netherlands, Belgium, France, Ireland, Luxembourg, Switzerland, Germany and Finland) while the remaining 7 countries (Austria, Denmark, Sweden, Norway, Hungary, Poland and Spain) have reported random cases in 2008 (Kuijper et al., 2008).



### **1.8 Control of *C. difficile* disease**

The basic strategy used to treat patients with CDAD is stopping the intake of any other antibiotics if possible. One of the recent studies found that 41% of patients failed treatment with metronidazole when other antibiotics were not stopped (Gould and McDonald, 2008). Metronidazole and vancomycin are considered the primary agents used to treat CDAD. Metronidazole treatment is preferred because it is less expensive and less likely to promote the selection of vancomycin-resistant *Enterococcus* spp. (Gould and McDonald, 2008). Gold and McDonald also report that some recent treatment failure of metronidazole has been reported especially in patients with severe complications. Metronidazole and vancomycin should be administered orally if possible and anti-peristaltic agents should be avoided. Early surgical operations sometimes improve the survival of patients with fulminate disease (Gould and McDonald, 2008).

The emergence of the hypervirulent strain has increased interest in *C. difficile* typing and stimulated the application of newer genotype-based methods. Faster typing techniques will enable the quicker detection of hypervirulent strains and the timely implantation of infection control strategies.

Improvement in the prevention and control of CDI armed by surveillance should be integrated into quality improvement efforts. Screening tests must be mandatory for all patients who develop diarrhoea after 48 hours of admission to a hospital. A stool test positive for toxigenic *C. difficile* or its toxins or colonoscopic and/or histological findings of PMC with the presence of symptoms (usually diarrhoea) are important points for case definition (Cohen et al., 2010).

In order to implement effective infection control precautions, infection control professionals and clinicians must be informed in a timely fashion when CDI cases have been detected (Cohen et al., 2010). Due to the possibility of CDI presenting in patients from residential aged care facilities or from the community, hospitals should also be alerted of the patient's history which may indicate a predisposition to CDI. Follow-up of patients who have one or more risk factors leading to CDI such as antibiotics exposure, old age or who have long hospital stays and testing of outpatients > 60 years of age who are suffering from diarrhoea should be taken into account (Australian Commission for Safety and Quality in Healthcare, 2010).

Hand hygiene plays an important role in the prevention of healthcare associated infection (HAI). A combination of educational programs and alcohol-based hand disinfection has revolutionised the practice of point of care hand hygiene. Reductions in HAI with methicillin-resistant *Staphylococcus aureus* (MRSA) have been reported to be related to an increase in the compliance to a hand hygiene program (Grayson et al., 2008, McLaws et al., 2009). Prevention of the transmission of *C. difficile* is also facilitated by hand hygiene (Gerding et al., 2008), but the best method of control is unknown. Several recent studies have shown that the extensive use of alcohol hand hygiene actually increases the spread of CDI (Boyce et al., 2006, Gordin et al., 2005). This is because *C. difficile* spores are resistant to alcohol hand hygiene products and soap and water is the preferred hand hygiene method (Boyce et al., 2006).

A main role of a surveillance programme is to identify new emerging strains of *C. difficile*. CDI caused by certain strain types, in particular ribotype 027, has been associated with a more severe course of disease, higher mortality and increased transmissibility between persons. Early detection of new emerging hypervirulent strains

is essential to control and prevent the spread of such clones. In new surveillance programmes, laboratories should culture for *C. difficile* from cases of severe CDI (including cases of PMC) and from cases in suspected outbreaks. The cultured isolates could then be characterised by ribotyping in a central reference laboratory. The culture and typing of *C. difficile* would be useful in investigating whether there are links between individual cases and in monitoring the spread of infection from particular strains of *C. difficile*.

### **1.9 Objectives and specific aims**

This study aims to trial a molecular technique for the rapid diagnosis of toxigenic *C. difficile* directly from stool samples. Secondly, we also aim to investigate a real-time PCR assay to identify those strains carrying the frameshift mutation ( $\Delta 117$  mutation) in the *tcdC* characteristic of hypervirulent strains. Thirdly we aim to develop a new method to track highly lethal hospital-acquired infections due to *C. difficile* based on the melt curve analysis of PCR products obtained from the ISR of *C. difficile*.

## **CHAPTER 2**

### **Development of a Multiplex Real-Time PCR for the Detection of Toxigenic *Clostridium difficile* and Subsequent Identification of the *tcdC* $\Delta$ 117 Mutation in Human Faecal Samples**

## 2.1 Introduction

Laboratory diagnosis of *C. difficile* should include testing of stool samples by isolation of *C. difficile* on culture or cytotoxicity testing as EIA methods for detection of toxins are insufficiently sensitive. PCR amplification of target genes is known to have very high specificity and sensitivity when compared to other pathogen detection methods (Louie et al., 2000). The advent of probe based real-time PCR has further increased the specificity and sensitivity of such assays. Probe based real-time PCR assays allow detection of targets much earlier during PCR cycling and negates the need for post-assay analysis of the PCR product. Therefore, probe based real-time PCR allows the acquisition of results in less time as compared to other diagnostic methods.

Persson et al. (2008) reported on a multiplex PCR assay for detection of *C. difficile* toxin genes and found that their PCR method was useful to characterise strains for the important toxin genes. Wroblewski et al. (2009) used a real-time PCR assay to detect the same genes and found they could also detect these genes directly from stool samples (Wroblewski et al., 2009). Recently de Boer *et al.* (2010) published a real-time PCR method that could discriminate between wild-type *tcdC* and the mutant form characteristic of ribotype 027. In order to perform PCR testing, it is important to select a DNA extraction method that will give reliable results.

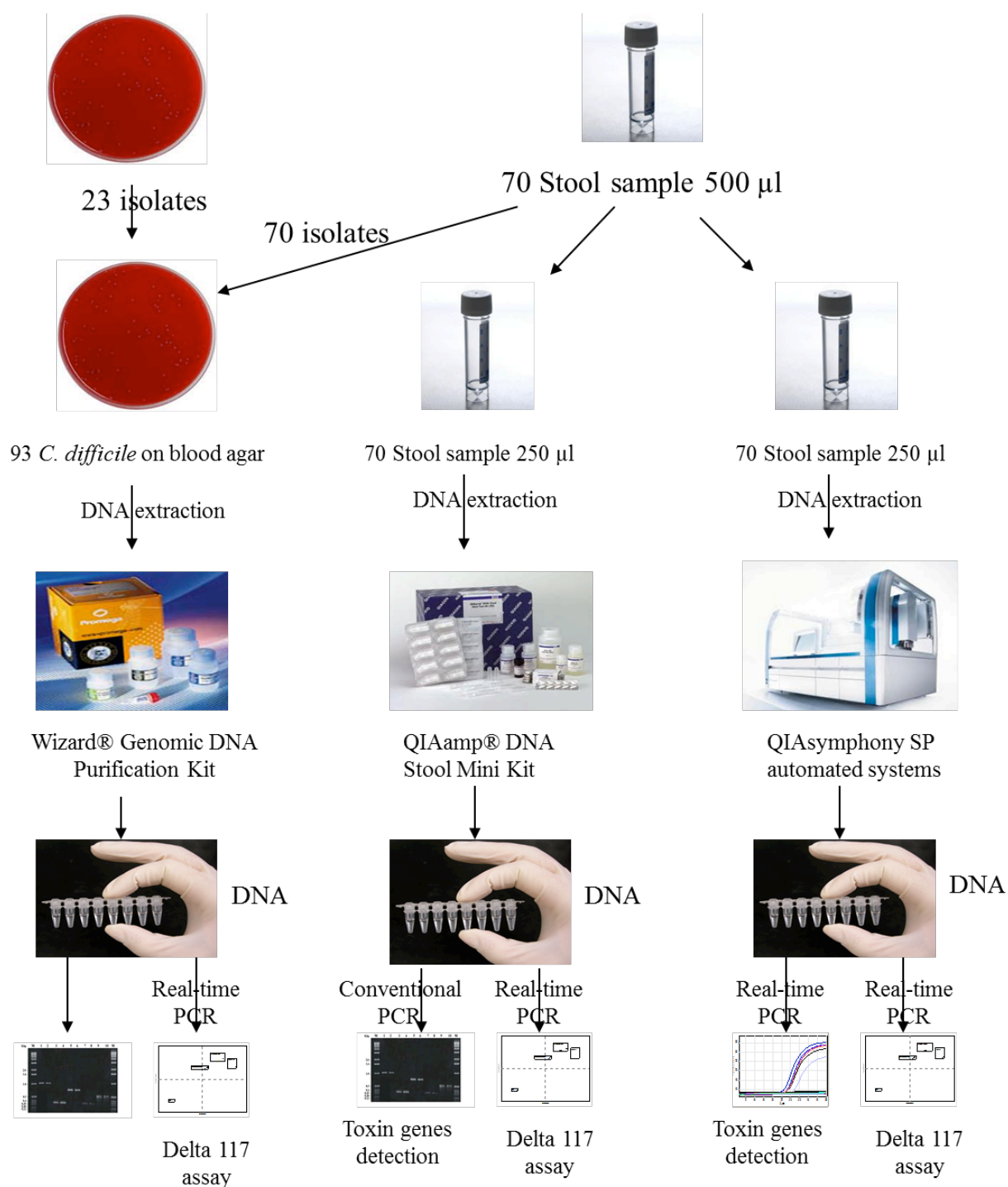
DNA extraction from pure cultures and stool samples using commercial kits is relatively time consuming, labour intensive and introduces the possibility of contamination. The robotic QIA Symphony SP can be used to avoid the issues associated with manual kits. Moreover, a robotic pipette can be used for aliquoting PCR master mix and DNA and thus decrease the pipetting errors that may occur with manual pipetting.

The research questions we aimed to answer in this part of the study were

1. Can published primers and probes for *C. difficile* toxin genes be improved by considering alignments with the latest published sequences?
2. Does real-time PCR perform as well as conventional PCR for stool DNA?
3. Does a deletion assay for the TcdC gene give similar results when used in the Rotor-Gene platform?
4. Does the use of a real-time multiplex assay for *C. difficile* toxin genes lead to reliable results?
5. Can an automated system (QIASymphony SP and a robotic pipette) be used to reliably detect *C. difficile* toxin genes and the  $\Delta 117$  mutation?

## 2.2 Materials and Methods

An overall flowchart for the testing is provided in Figure 2.1. Details are provided in the following sections.



**Figure 2.1 Flowchart of sample testing**

### 2.2.1 Clinical specimens and bacterial isolates

Stool samples (70) with positive results for *C. difficile* isolation were collected from the Microbiology Department at Austin Health (Melbourne, Australia) during the period between May 2009 and May 2010. Approximately 1 ml of each sample was frozen at -80°C. These stool samples had also been previously tested for *C. difficile* cytotoxin in cell culture cytotoxicity. *C. difficile* was re-isolated from samples by culturing the frozen stool specimens onto *C. difficile* selective media (Cycloserine Cefoxitin Blood Agar) and the plates were incubated for 48 h at 37°C in an anaerobic chamber. A colony showing typical *C. difficile* morphology was then sub-cultured onto the same media and incubated as previously stated. The isolates were confirmed as *C. difficile* through colonial morphology and characteristic odour, Gram stain and CD Agglutination Test (OXOID, Adelaide, Australia) performed according to the manufacturer's instructions. A further 23 isolates were forwarded from Austin Health directly after isolation from faecal samples. 32 of the 70 stool samples were reported to be positive in the cytotoxicity assay performed by Austin Health. Five reference strains were included as controls: *C. difficile* ATCC9689 and four *C. difficile* PCR ribotype 027. The control strains were: CD196, the original non-epidemic strain first isolated in Paris in 1985 (Popoff et al., 1988); R20291, a representative strain from the 2006 Stoke Mandeville outbreak in the United Kingdom (Stabler et al., 2009); M7404 provided by J. Pepin to Monash University, Australia (Carter et al., 2007) and a representative of the Canadian outbreak in 2005 (Loo et al., 2005); and KI, a strain locally acquired in Melbourne, Australia causing severe infection (Richards et al., 2011). These strains were kindly supplied by Dena Lyras and Glen Carter, Department of Microbiology, Monash University.



### **2.2.2 Extraction of DNA from bacterial isolates**

DNA was extracted from isolated *C. difficile* colonies using a Wizard® Genomic DNA Purification Kit (Promega, San Diego, USA). Briefly, ten colonies were resuspended thoroughly in 480 µl of 50 mM EDTA. 60 µl of 20 mg/ml lysozyme was added to the cell pellet to weaken the cell wall. The cell suspensions were incubated at 37°C for 45 minute (min) and then centrifuged at 13,000 x g for 3 min. The supernatant was then removed and 600 µl of Nuclei Lysis Solution was added and then samples incubated at 80°C for 5 min then cooled to room temperature. 200 µl of Protein Precipitation Solution was then added to the solution and vortexed vigorously for 20 sec. Samples were then incubated on ice for 5 min and centrifuged at 13,000 x g for 4 min. The supernatant containing the DNA was then transferred to a clean 1.5 ml microcentrifuge tube containing 600 µl of room temperature isopropanol. The samples were then mixed gently until thread-like strands of DNA formed a visible mass and then the samples were centrifuged at 13,000 x g for 3 min. The supernatant was carefully poured off and the tube was drained on clean absorbent paper. 600 µl of room temperature 70% ethanol was then added and the tube gently inverted around ten times to wash the DNA pellet. The samples were centrifuged at 13,000 x g for 3 min and the ethanol carefully aspirated. The tubes were then drained on clean absorbent paper and the pellet allowed to air dry for 15 min. 100 µl of DNA Rehydration Solution was added to the tubes and the DNA rehydrated by incubating at 65°C for 1 h with the solution mixed periodically. The DNA solution was then cooled to room temperature and stored at 4°C.

### **2.2.3 Extraction of DNA directly from stool samples**

Stool samples were centrifuged at 13,000 x g for 4 min and the supernatant was removed in order to leave 500 µl including the sediment. The sediment was then re-suspended in the 500 µl and the total was divided into two parts (each 250 µl). DNA was extracted using

QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) on 250 µl of the faecal suspension and QIASymphony SP automated systems (QIAGEN) on the remaining 250 µl of the faecal suspension. The QIAamp® DNA Stool Mini Kit was used according to the manufacturer's instructions. Briefly, stool samples were homogenized in 1.5 ml of buffer ASL and heated at 95°C for 5 min to lyse bacterial cells. After removal of potential inhibitors by incubation with an InhibitEx tablet (QIAGEN) the lysates were treated with 15 µl proteinase K and 200 µl buffer AL at 70°C for 10 min to remove protein and polysaccharides. DNA was precipitated by adding 200 µl of ethanol (97%) to the lysate and then applied to a column provided in the kit. This was followed by washes with 500 µl of buffers AW1 and AW2 and centrifuged at full speed for 3 min. The QIAamp spin column containing the filtrate was discarded and sediments dissolved in 200 µl buffer AE. For the DNA extracted by The QIASymphony, the stool samples were prepared as described above. 250 µl of each sample was loaded into the machine and DNA extraction was performed using the QIASymphony Virus/Bacteria Mini Kit (QIAGEN) using the QIASymphony “Complex200 V3 default IC” extraction protocol which extracted DNA from 200 µl of the 250 µl presented. The resulting DNA was eluted in 110 µl of elution buffer.

#### **2.2.4 Molecular techniques**

Multiplex PCR was performed for *tcdA*, *tcdB*, *cdtA*, *cdtB* and 16s rDNA, according to the method of Persson et al. (2008). After the initial results showed weak bands for detection of toxin genes from some isolates the method was changed to a monoplex PCR for the detection of *tcdC* and 16S rDNA and 2 plex PCR for the detection of the *C. difficile* toxin genes, *tcdA* and *tcdB* and for *C. difficile* binary toxin genes *cdtA* and *cdtB*. After the initial results showed that the 2 plex PCR in stool was unable to detect *tcdA* or *tcdB* from some stool samples the method was changed to a monoplex PCR.

Specific amplification of a segment of 16S rDNA was used as a positive control to determine whether non-specific inhibition of PCR reactions was occurring and to verify the quality of the genomic DNA template. Primers were manufactured by GeneWorks (Thebarton, Australia). A robotic pipette (Castro 1200, QIAGEN) was used for loading the Master Mix and DNA to decrease pipetting errors that may occur with manual pipetting.

#### **2.2.4.1 Conventional PCR**

Conventional PCR for the detection of *tcdA*, *tcdB*, *cdtA*, *cdtB*, 16S rDNA and *tcdC* was performed to identify the presence of toxigenic and non-toxigenic *C. difficile* in stool samples using a Rotor-Gene 6000 (QIAGEN). 5 µl DNA extracted from isolated *C. difficile* colonies was used for the conventional PCR in a 50 µl reaction volume, which was composed of 5 µl buffer 2 (PE), 2.5 mM MgCl<sub>2</sub>, 16 µM dNTPs and 0.5 µM from each forward and reverse primers as described by Persson et al. (2008) and 2U of AmpliTaq Gold (Perkin Elmer).

The conventional PCR for detection of *C. difficile* genes was also performed using DNA extracted directly from stool samples. A 50 µl reaction volume composed of 25 µl of HotStarTaq plus Master Mix (QIAGEN), 0.5 µM for each forward and reverse primers as described by Persson et al. (2008), 0.5 µl BSA and 20 µl of the genomic template DNA was used per reaction. The thermocycler conditions for the conventional PCR using isolate DNA were 10 min at 94°C, followed by 35 cycles of 50 s at 94°C, 40 s at 54°C and 50 s at 72°C and a final extension of 3 min at 72°C. After the initial results showed some weak bands for detection of toxin genes from stool samples by conventional PCR, the annealing temperature and cycle number were modified from that reported by Persson et al. (2008) to give better reproducibility. The final cycling conditions for stool DNA were 10 min at

94°C, followed by 45 cycles of 50 s at 94°C, 40 s at 57°C and 50 s at 72°C, and a final extension of 3 min at 72°C. Amplification of *tcdC* was performed using isolate DNA with both forward and reverse primers as described by Persson et al. (2008) in a 50 µl reaction volume. The composition of the reaction was as described for testing isolate DNA for the above listed genes. Thermocycle conditions were 6 min at 94°C, followed by 35 cycles consisting of 50 s at 94°C, 40 s at 47°C and 50 s at 72°C, and a final extension at 72°C for 3 min.

The PCR products were separated on preformed 2% TAE agarose gels (AMRESCO, Solon, USA) by electrophoresis using a gel dock. The gel was completely immersed in fresh room temperature TAE buffer, and 10 µl of PCR product from each PCR reaction was then mixed with 2.5 µl EZ-Vision® DNA Dye (AMRESCO, Solon, USA) as loading buffer and loaded sequentially into the wells. Marker VI DNA (Roche, Indianapolis, USA) was used in the first and last wells. A constant 130 volt charge was applied across the gel for 30 min, and the resulting electrophoresis banding patterns were visualized under 280nm ultraviolet (UV) light. Each gel picture was recorded using Fluor-S™ MultiImager (Bio-Rad, Hercules, USA).

The PCR products obtained from amplification of *tcdC* were sequenced when the band size was less than the predicted 475 bp for wild-type isolates. Each isolate requiring sequencing was amplified 5 times as described above for *tcdC* detection (i.e. 5x50 µl PCR reactions per sample) to provide sufficient amounts of product for purification. Purification and concentration was performed using a Wizard PCR Preps DNA Purification System (Promega) according to the manufacturer's instructions. Sequencing was performed by the Gandel Charitable Trust Sequencing Center (Monash Health Research Precinct, Melbourne, Australia) on an ABI Prism automated DNA sequencer. On receipt of the sequences,

analysis was performed using Geneious (version 5.0.4) DNA analysis program (Biomatters, Auckland, New Zealand).

#### **2.2.4.2 Real-time PCR primer and probe design**

The primers and probes used in this study were modified from Wroblewski et al., (2009) after examining published sequences for target genes (Table 2.1). Primer and probe sets targeted *C. difficile* enterotoxin A gene (*tcdA*), cytotoxin B gene (*tcdB*), and binary toxin genes A and B (*cdtA* and *cdtB*) respectively. The modified primers and probes are presented in Table 2.1. An internal control (IC) was included during the QIA Symphony SP automated DNA extraction to monitor the efficiency of sample preparation and downstream assay. The DNA template used as an IC is a synthetic oligonucleotide derived to be unlikely to resemble existing bacterial sequences and is added into the specimen by the QIA Symphony SP prior to DNA extraction. The sequence for the IC was:

GTCCTCACACCAGTTGCCGCGCAAAGTATGTGGAATGTTAACACACCCACACC  
ACACCCACACACGTGT TGGATC AATTTCGAGATGCGAGCTGCCAAGC.

A negative result for the IC after PCR of samples indicates failure of DNA extraction (ruled out if DNA is visualised on electrophoresis) or amplification inhibition.

**Table 2.1** Primer and probe sequences used in real-time multiplex PCR assay and Deletion assay ( $\Delta 117$ ). The letters in the red colour are the modified nucleotides from Wroblewski et al. (2009).

Target	Sequence name	Sequence (5'-3')	Product length (bp)	Region	Reference
<i>tcdA</i>	CD- <i>tcdA</i> -F*	CAGRGCTAATAGYTTGTTTACAGAACA	143	717-859	Wroblewski et al., 2009
	CD- <i>tcdA</i> -R	CAACATCTAAATATACTCCGCCAAAA			Wroblewski et al., 2009
	CD- <i>tcdA</i> -MGB1*	5'd CAL Flour Red 610 GCAGCTAAATTYCCACGATTTAACAACCTCCTGAYTATAA-BHQ-2 3'			Wroblewski et al., 2009
<i>tcdB</i>	CD- <i>tcdB</i> -F*	AGCAGTTGAMTATAGYGGTTTAGTTAGAGTTN	144	6552-6695	Wroblewski et al., 2009
	CD- <i>tcdB</i> -R*	YTAGCTTTTTTAGTTTCTGGATYGAA			Wroblewski et al., 2009
	CD- <i>tcdB</i> -MGB*	5' d Quasar 705-CAWCCAGTYTCAATTGTATATGWTCTCCA-BHQ-2 3'			Wroblewski et al., 2009
<i>cdtA</i>	CD- <i>cdtA</i> -F	GATCTGGTCCTCAAGAATTTGGTT	103	1051-1153	Wroblewski et al., 2009
	CD- <i>cdtA</i> -R	GCTTGTCCTTCCCATTTGATT			Wroblewski et al., 2009
	CD- <i>cdtA</i> -MGB	5' d FAM-AACTCTTACTTCCCCTGAAT-BHQ-1 3'			Wroblewski et al., 2009
<i>cdtB</i>	CD- <i>cdtB</i> -F	AAAAGCTTCAGGTTCTTTTGACAAG	132	837-968	Wroblewski et al., 2009
	CD- <i>cdtB</i> -R	TGATCAGTAGAGGCATGTTTCATTTG			Wroblewski et al., 2009
	CD- <i>cdtB</i> -MGB*	5' d CAL Fluor Orange 560 -CAAGAGATCCGTTAGTTGCAGCRTATCCAATTGT-BHQ-1 3'			Wroblewski et al., 2009
<i>tcdC</i> ( $\Delta 117$ )	CD- <i>tcdC</i> -F	GCA CAA AGG RTA TTG CTC TAC TGG	70	83-152	de Boer et al, (2010)
	CD- <i>tcdC</i> -R1	AGC TGG TGA GGA TAT ATT GCC AA			de Boer et al, (2010)
	CD- <i>tcdC</i> -R2	CAA GAT GGT GAG GAT ATA TTG CCA			de Boer et al, (2010)
	CD- <i>tcdC</i> wt-MGB	6FAM-AAA CAC RCC HAA AAT AA-NFQ-MGBNFQ			de Boer et al, (2010)
	CD- <i>tcdC</i> mut-MGB	VIC-AAA CAC RCC AAA ATA A-NFQ-MGBNFQ			de Boer et al, (2010)
(IC)	Internal Control-F	GTG CTC ACACCAGTTGCCGC	100		
	Internal Control-R	GCTTGGCAGCTCGCATCTCG			
	Internal Control-P	5' [cy5]ATTGTGTGGGTGTGGTGTGGGTGTGTGC[BHQ3] 3'			

#### **2.2.4.3 Real-time multiplex PCR assay**

The real-time PCR assay was performed on a Rotor-Gene 6000 series 5 plex (QIAGEN) in a 72-well optical plate format. This machine has five fixed path lengths and a separate high intensity light-emitting diode (LED) excitation source and matching emission filter for five channels. Orange 585nm/610nm, Crimson 670nm/710nm, Green 470nm/510nm, yellow 530nm/555nm and Red 625nm/660nm channels were used for *tcdA*, *tcdB*, *cdtA*, and *cdtB* and Internal Control probes respectively. For testing of the isolates, each 26.5 µl reaction mixture consisted of 12.5 µl of mastermix (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP's), 1.5 µM SYTO 9 dye (Invitrogen, CA, USA) and 0.025 U/ µl reaction volume AmpliTaq Gold DNA polymerase (Applied Biosystems, Life Technologies, CA, USA) and 0.4 µM of each primer, 0.15 µM of each probe and 10 µl genomic DNA. Reactions were run under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Data was acquired and analysed with the Rotor-Gene 6000 series software (QIAGEN). PCR runs were analyzed using a fixed threshold of 0.05. A PCR run was considered valid when the no template control (NTC) was negative, and the IC was positive with a threshold cycle (Ct) of less than 40 cycles. A real-time PCR was considered inhibited when the Ct value for the IC exceeded 34.91 cycles (i.e. the mean Ct value for uninhibited specimens+2 standard deviations).

#### **2.2.4.4 Deletion assay (delta 117 detection)**

A delta 117 PCR assay ( $\Delta$ 117) (de Boer et al., 2010) for the detection of isolates carrying the  $\Delta$ 117 *tcdC* mutation was performed on extracted DNA from isolates and samples. The  $\Delta$ 117 assay utilizes two TaqMan MGB probes, a wild type (WT) probe and a mutant (MUT  $\Delta$ 117) probe respectively that both can hybridize with the part of the *tcdC* sequence that flanks the 1 bp deletion at position 117. Isolates that do not carry the 1 bp deletion will give

a stronger signal with the WT probe, while 027/NAP1 isolates will do so with the MUT  $\Delta 117$  probe. The  $\Delta 117$  PCR assay primer/probe set was used as described by de Boer et al. (2010) (Table 2.1).

The  $\Delta 117$  PCR assay was performed on 98 *C. difficile* (93 clinical isolates and five reference control strains) and 70 stool samples using TaqMan Genotyping Master Mix (QIAGEN). DNA used was from isolates and stools (manual extraction and robotic extraction). Each 20  $\mu$ l reaction consisted of 5 ng of extracted DNA, 10  $\mu$ l TaqMan Genotyping Master Mix (Applied Biosystems, Foster, USA) and 0.4  $\mu$ M of each primer, 0.1  $\mu$ M of each probe. Reactions were run under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min.

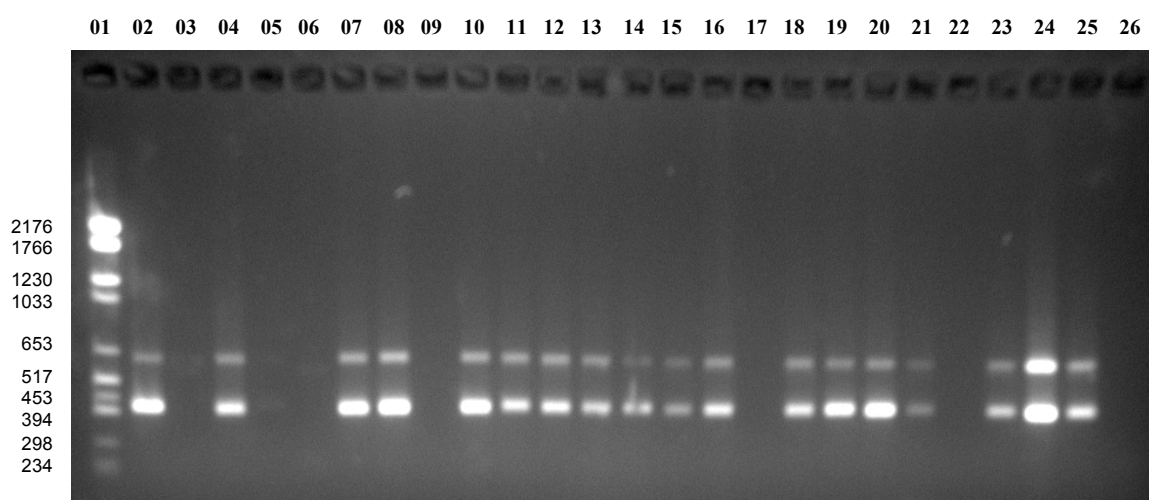
The real-time TaqMan PCR deletion assay was carried out on the Rotor-Gene 6000 series 2 plex (QIAGEN) with the use of scatter plotting software (QIAGEN) for analysis of results.



## 2.3 Results

### 2.3.1 Analysis of Conventional PCR using isolates DNA

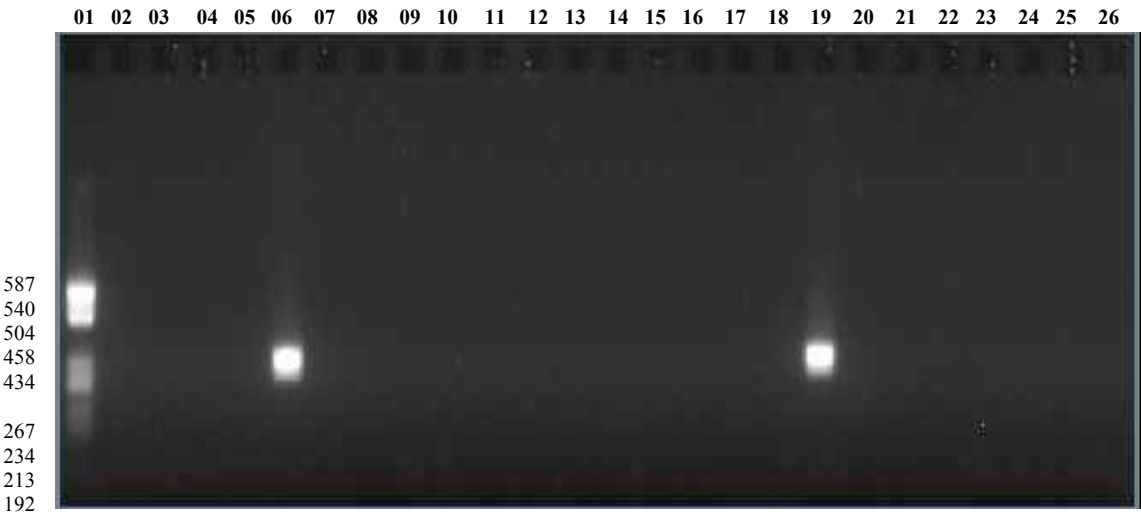
All isolates were confirmed as *C. difficile* through colonial morphology, characteristic odour, Gram stain and CD Agglutination Test (OXOID). After the initial testing of the multiplex PCR showed some weak bands (Appendix A1) the method was changed to a 2-plex PCR for the detection of the *C. difficile* toxin genes, *tcdA* and *tcdB* and for *C. difficile* binary toxin genes (*cdtA* and *cdtB*) and a monoplex PCR conducted for *tcdC* and 16S rDNA. All 93 clinical isolates produced a PCR product in the monoplex for 16s rDNA. All isolates producing a *tcdA* band also produced a *tcdB* band. PCR amplification results of *tcdA* and *tcdB* of 24 isolates are shown in Figure 2.2. The *tcdA/tcdB* bands for other isolates are presented in Appendix A (Figures A2, A3, A4, and A5). The number of isolates with toxin genes and combination of toxin genes is summarized in Table 2.2.



**Figure 2.2** Amplification of *tcdA* (629 bp) and *tcdB* (410 bp) by 2-plex conventional PCR in 24 *C. difficile* isolates. Lane 1 is DNA marker VI (154-2176 bp); lanes 2-25 representing *C. difficile* isolates number 01-24; lane 26 is a NTC.

Amplification of *cdtA* and *cdtB* showed that seven isolates out of a total of 93 tested positive. It was not possible to show that both toxin genes were amplified because the band for *cdtA* was a similar size to that of *cdtB* and thus is shown as a single strong band (Figure

2.3). For the purpose of identifying the presence of binary toxin genes it was decided that the presence of a single strong band around 260 bp would be sufficient. The result of two positive isolates is shown in Figure 2.3. The *cdtA/cdtB* bands for other isolates are presented in Appendix A (Figures A6, A7, A8 and A9).



**Figure 2.3** Amplification of *cdtA* (221 bp) and *cdtB* (262 bp) by 2-plex conventional PCR. Lanes 1 is DNA marker V (8-587 bp); lanes 2-25 representing *C. difficile* isolates number 25-48; lane 26 is a NTC.

The results of *tcdA*, *tcdB* and binary toxin (*cdtA* and *cdtB*) detection by conventional PCR using isolate DNA on 93 isolates are summarised in Table 2.2

**Table 2.2** Conventional PCR results for toxin genes in 93 clinical isolates.

<i>tcdA/tcdB</i>	<i>cdtA/cdtB</i>	<i>tcdC</i> nucleotide Deletions (≥ 1 bp)	<i>tcdC</i> stop codon at 184 bp	No. of isolates found
-	-	-	-	34
+	-	-	-	50
+	-	18 bp	-	2
+	+	54 bp	-	1
+	+	21 bp	-	3
+	+	39 bp	+ / -*	2
+	+	Δ117 and 18 bp	-	1

Total 93

\* Both isolates have 39 bp deletions in *tcdC* but only one of them contained a C-T transition at position 184 bp.

As previously showed in materials and methods Figure 2.1 the toxin gene results of the 93 isolates in Table 2.2 comes from 23 isolates forwarded from Austin Health and 70 isolates were from 70 stool samples from the Microbiology Department at Austin Health. The results of toxin genes for these 70 isolates where corresponding stool samples were also available to be tested represented in Table 2.3.

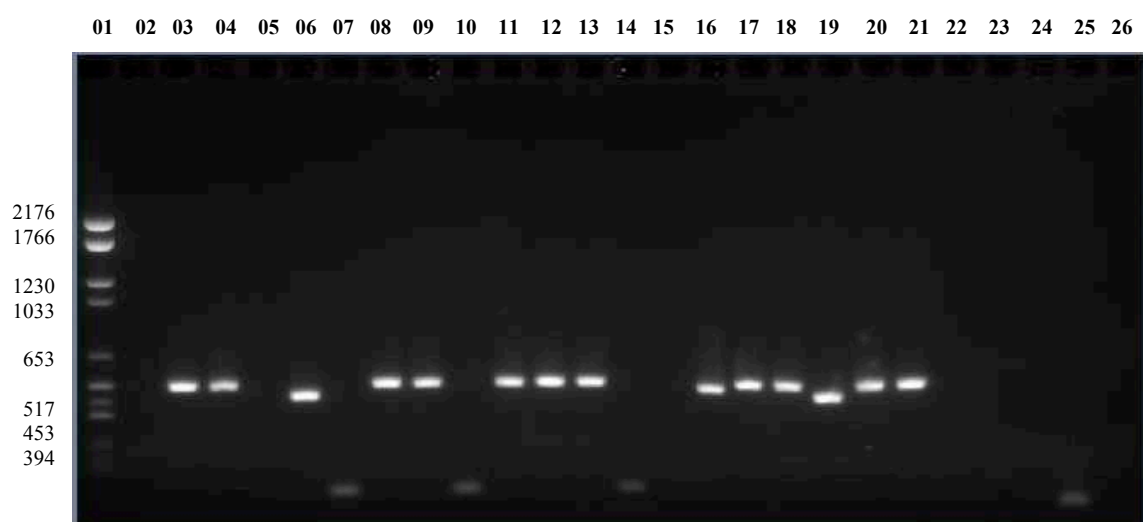
**Table 2.3** Conventional PCR results for toxin genes in 70 clinical isolates where stool was also available for later testing.

CTA	<i>tcdA/tcdB</i>	<i>cdtA/cdtB</i>	No. of isolates found
+	+	+	5 (7.2%)
+	+	-	24 (34.3%)
+	-	-	4 (5.7%)
-	-	-	21 (30%)
-	+	+	1 (1.4%)
-	+	-	15 (21.4%)

Total 70

From Table 2.3, PCR for *tcdA/tcdB* was in agreement with stool CTA in 71.5% of samples. In four samples cytotoxicity of stool was reported positive, yet the *tcdA/tcdB* results were negative. 22.8% of isolates were positive for *tcdA/tcdB* but were reported negative in cytotoxicity testing. 8.6% of the 70 isolates were binary toxin gene positive.

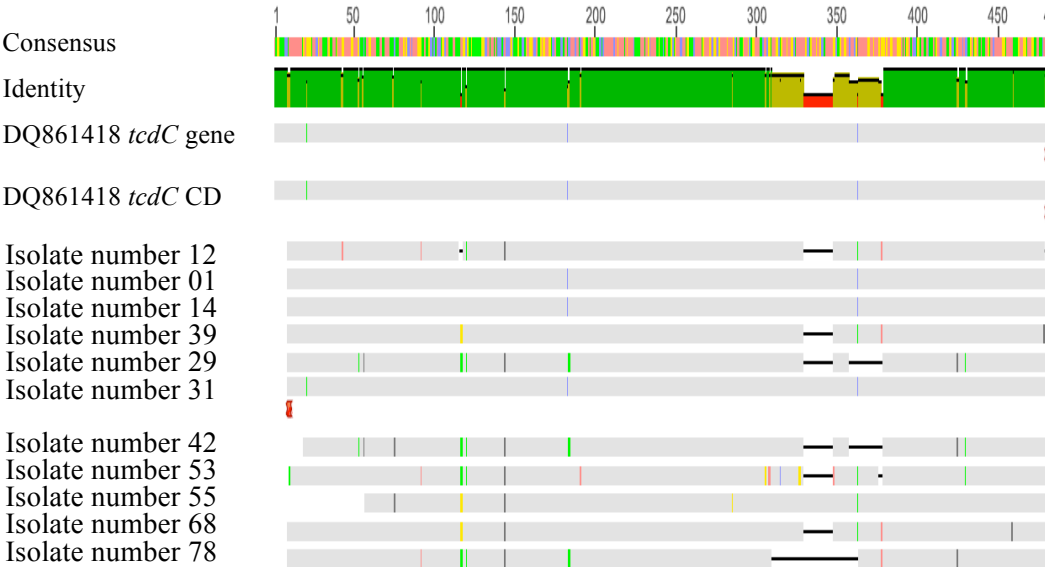
PCR products obtained from the amplification of *tcdC* of 93 isolates showed an amplicon of around 475 in 84 (90.3%) isolates. Nine isolates (9.7%) (Isolates 12, 29, 39, 42, 53, 54, 60, 68 and 78) showed a PCR product for *tcdC* of less than 475 bp. Figure 2.4 shows three of these isolates in lanes 6, 16 and 19. Results of the remaining isolates are shown in Appendix A (Figures A10, A11, A12 and A13).



**Figure 2.4** Amplification of *tcdC* (475 bp) by conventional PCR. Lane 1 is DNA marker VI (154-2176 bp); lanes 2-25 representing *C. difficile* isolates number 25-48; lane 26 is a NTC.

Sequencing of *tcdC* was performed for seven isolates with PCR products less than the predicted 475 bp in wild-type isolates. Four isolates numbered as (01, 14, 31 and 55) with normal *tcdC* band size (475 bp) were also sequenced to compare with these seven isolates. The reference sequence DQ861418 was extracted from GenBank to use as a reference guide for alignment (Figure 2.5). Sequencing of these 11 isolates revealed that six isolates

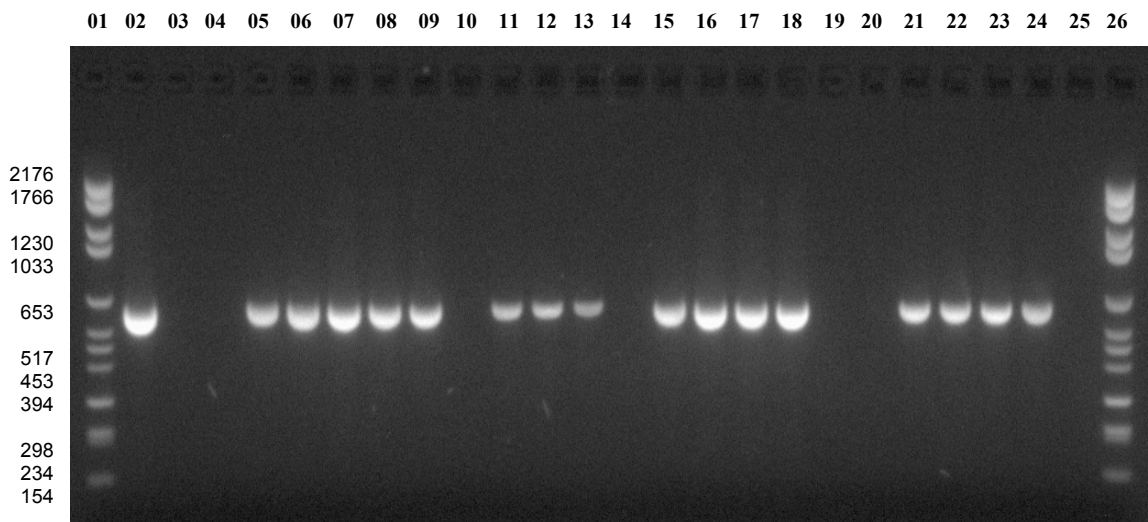
contained gene deletions of 18, 21, 39 or 54 bp and that one of these isolates (isolate number 29 shown in Figure 2.5) contained a C – T transition at position 184 bp which would introduce a premature stop codon. The 39 bp deletion was observed in isolate number 29 (sequence Figure 2.5) and isolate number 42 (sequence Figure 2.5). An 18 bp deletion was observed in isolates number 39 (sequence Figure 2.5) and isolate number 68 (sequence Figure 2.5). Isolate number 53 (sequence Figure 2.5) had a 21 bp deletion and isolate number 78 (sequence Figure 2.5) had a 54 bp deletion. Isolate number 12 was found to have a single nucleotide deletion at position 117 ( $\Delta$ 117 sequence Figure 2.5). This deletion has been described by MacCannell (2006) and introduces a frameshift that will result in a nonsense mutation at position 196 and a radical alteration in the preceding 26 deduced amino acid residues. The predicted *tcdC* gene product is truncated from 232 to 65 amino acid residues. This isolate also contained an 18 bp deletion between nucleotides 330 to 347 which is identical to that previously described by MacCannell (2006) and characteristic of the 18 bp deletion in BI/NAP1/ribotype 027. 0923384942



**Figure 2.5** Comparison of *tcdC* nucleotide sequences obtained from sequenced PCR products of 11 isolates to the published sequence for the reference strain DQ861418. Grey lines and dashes indicate identical bases and deletions, respectively. Coloured lines in the sequenced PCR products (lines 3-13) indicate bases different to the consensus sequence listed at the top of the Figure.

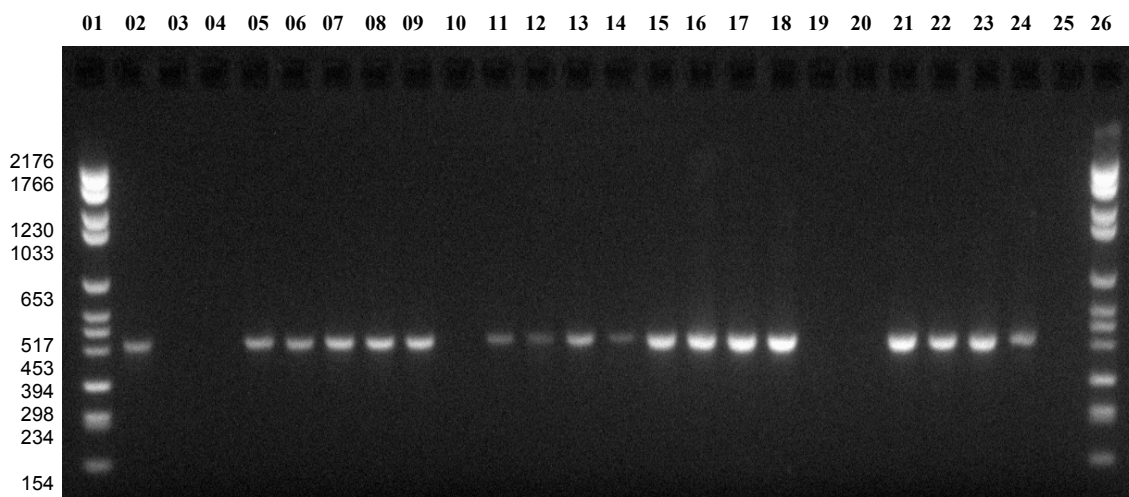
### 2.3.2 Analysis of conventional PCR using stool DNA

Conventional PCR using DNA extracted directly from 70 stool samples by QIAamp® DNA Stool Mini Kit (QIAGEN) was performed to detect *C. difficile* toxin genes *tcdA/tcdB* and *cdtA/cdtB* as well as 16S rDNA as an internal PCR control. The original annealing temperature of 54°C as reported in Persson et al. (2008) showed weak bands in initial testing. After further testing, 57°C was used for annealing. The result of the internal PCR control 16S rDNA was positive for all 70 stool samples. Results of *tcdA* of 23 stool samples are shown in Figure 2.6. Results of remaining isolates are shown in Appendix B (Figures B1 and B2).



**Figure 2.6** Amplification of *tcdA* resulting in a 629 bp product by conventional PCR using stool DNA. Lanes 1 and 26 are DNA molecular weight marker VI (154-2176 bp); Lanes 02-24 representing stool samples number (1, 4, 5, 12, 13, 17, 18, 19, 22, 25, 26, 27, 29, 31, 32, 35, 36, 37, 38, 39, 42, 43 and 51); lane 25 negative control.

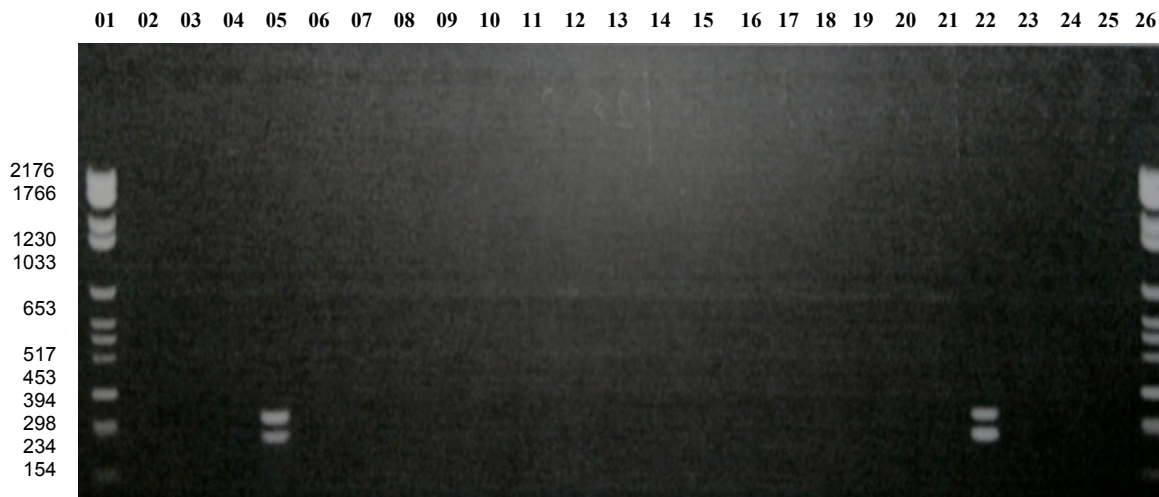
Results of *tcdB* of 23 stool samples are shown in Figure 2.7. Results of remaining isolates are shown in Appendix B (Figures B3 and B4).



**Figure 2.7** PCR products from amplification of *tcdB* (410 bp) by conventional PCR using stool DNA. Lanes 1 and 26 are DNA molecular weight marker VI (154-2176 bp); Lanes 02-24 representing stool samples number (1, 4, 5, 12, 13, 17, 18, 19, 22, 25, 26, 27, 29, 31, 32, 35, 36, 37, 38, 39, 42, 43 and 51); lane 25 negative control.

Many *tcdB* bands were lower in intensity than those obtained by *tcdA* PCR. However, *tcdB* PCR was able to detect a PCR product for sample number 29 (lane 14 Figure 2.7) whereas the *tcdA* PCR showed no band for this samples (lane 14 Figure 2.6). This sample was confirmed *tcdA/tcdB* positive in a repeat run.

PCR for *cdtA* and *cdtB* was performed on the 70 stool DNA extracts. Clear separation of the PCR products was obtained as shown in Figure 2.8. The result of two positive isolates is shown in Figure 2.8. The *cdtA/cdtB* bands for other isolates are presented in Appendix B (Figures B5 and B6).



**Figure 2.8** Amplification of *cdtA* 221 bp products and *cdtB* 262 bp products by conventional PCR using stool DNA in 24 stool sample. Lanes 1 and 26 are DNA marker VI (154-2176 bp); lanes 2-25 representing stool samples number (1, 4, 5, 12, 13, 17, 18, 19, 22, 25, 26, 27, 29, 31, 32, 35, 36, 37, 38, 39, 42, 43 and 51); lane 26 is a NTC.

The results of testing for *tcdA/tcdB* and *cdtA/cdtB* for the 70 stool samples is shown in Table 2.4 and compared to the CTA results reported on these stool samples.

**Table 2.4** PCR and CTA result of the 70 samples tested by conventional PCR using stool DNA

CTA	<i>tcdA/tcdB</i>	<i>cdtA/cdtB</i>	No. of samples found
+	+	+	4 (5.7%)
+	+	–	26 (37.1%)
+	–	–	3 (4.2%)
–	–	–	25 (35.7)
–	+	+	1 (1.4%)
–	+	–	11 (15.7%)

From Table 2.4, PCR for *tcdA/tcdB* was in agreement with stool CTA in 78.5% of samples. In three samples cytotoxicity of stool was reported positive, yet the *tcdA/tcdB* results were negative. 17.1% of isolates were positive for *tcdA/tcdB* but were reported negative in cytotoxicity testing. 7.1% of the 70 isolates were binary toxin gene positive.



### 2.3.3 Validation of the use of stool sample for testing of *C. difficile* toxin genes by PCR

To improve the speed of diagnostic testing for *C. difficile* it is important to be able to validate that testing for toxin genes on stool samples performs as well as testing on isolates. The results of 70 stool samples tested for *tcdA*, *tcdB* and binary toxin (*cdtA* and *cdtB*) by conventional PCR assay using DNA extracted from pure culture were compared to the results obtained by testing from the same stool using the same primers and DNA extracted directly from the same stool samples. The results showed that the 37 stool samples positive by conventional PCR using stool DNA were also positive by conventional PCR using isolate DNA. Twenty isolates were negative by conventional PCR using isolate DNA were also confirmed negative by conventional PCR using stool DNA (Table 2.5). The conventional PCR using DNA extracted directly from stool sample was positive in 5 additional samples and 8 samples were false negatives. If we use the PCR of isolate DNA as the gold standard then the sensitivity and specificity of the PCR on stool DNA is 82% and 80% respectively.

**Table 2.5** Comparison of conventional PCR (stool DNA) with conventional PCR (isolate DNA)

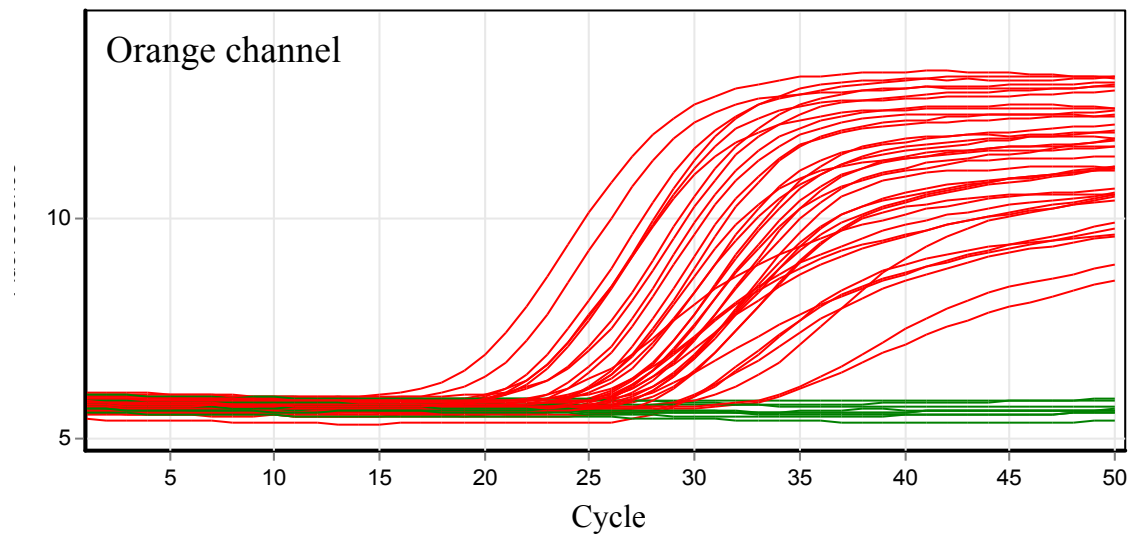
Assay	No. of samples included	Results	PCR(isolate DNA)		Sensitivity (%)	Specificity (%)
			Positive	Negative		
PCR (stool DNA)	70	Positive	37	5	82%	80%
		Negative	8	20		

Interestingly, although PCR of stool DNA failed to detect toxin genes in eight samples that were positive by testing isolate DNA, a similar number (5) were found to be positive in stool DNA but negative by testing isolates for toxin genes. Since testing of stool DNA had

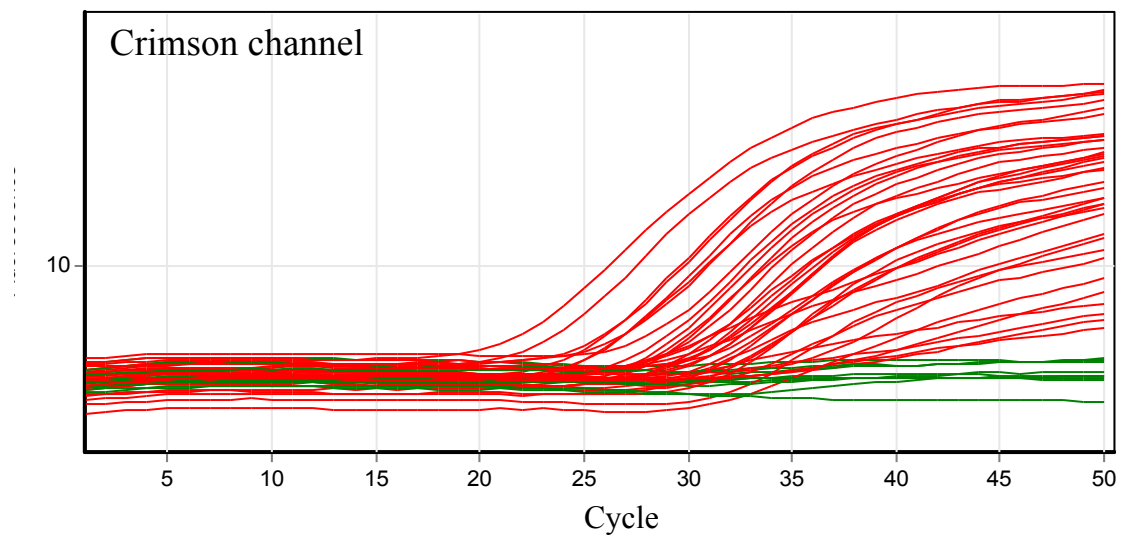
values not less than 80% for both sensitivity and specificity, it was decided that this would be the benchmark to compare the performance of real-time PCR.

#### **2.3.4 Analysis of multiplex real-time PCR assay using stool DNA**

A multiplex real-time PCR assay for detection of *C. difficile* toxin genes *tcdA*, *tcdB*, *cdtA* and *cdtB* was performed on a Rotor-Gene 6000 series 5 plex (QIAGEN) in a 72-well optical plate format using DNA extracted directly from 70 stool samples by QIAasymphony SP automated systems (QIAGEN). The results of 45 samples tested by this assay for detection of *tcdA*, *tcdB*, *cdtA* and *cdtB* are shown in Figures 2.9, 2.10, 2.11 and 2.12 respectively. Results of the remaining samples (46 – 70) are shown in Appendix C (Figures C1, C2, C3 and C4). The internal control was positive in all 70 stool samples and the Ct value for the IC for all samples was less than 34.91 as shown in Appendix C (Figures C5 and C6). The toxin gene profile of these 70 stool samples detected by this assay is summarized in Table 2.6 and is compared to the result of cytotoxicity testing of stool. There was complete agreement between the 50 samples that were *tcdA* positive and *tcdB* positive by real-time PCR. This was also found for the six samples positive for *cdtA* and *cdtB*. The mean Ct value for *tcdB* (Figure 2.10) was 29.36 (SD = 3.8) which was higher than the Ct value for *tcdA* which was 26.4 (SD = 3.8) (Figure 2.9).

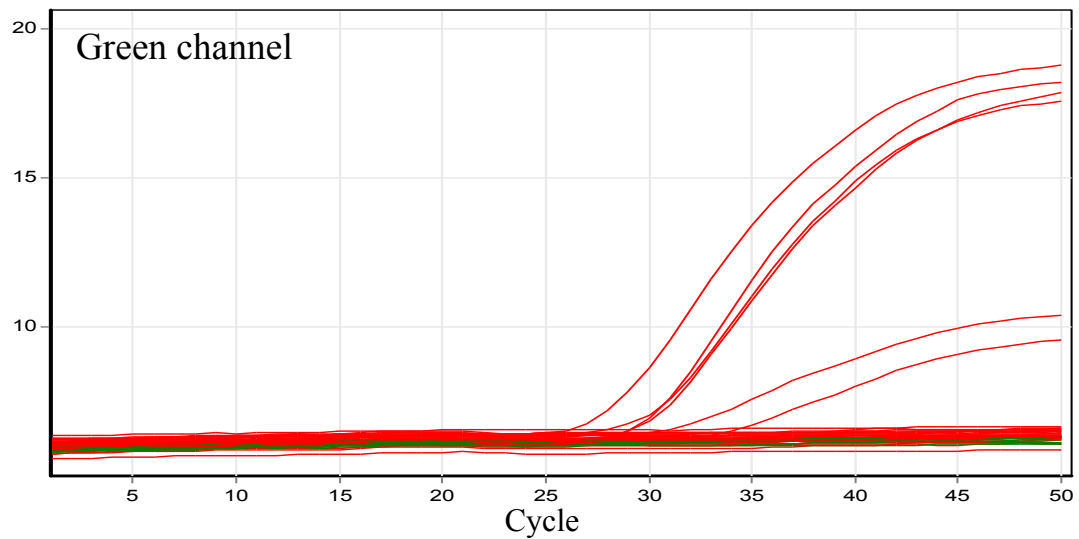


**Figure 2.9** Amplification of *tcdA* for samples 1-45 by multiplex real-time PCR. Red curves were *tcdA* positive samples while the green lines were *tcdA* negative samples.

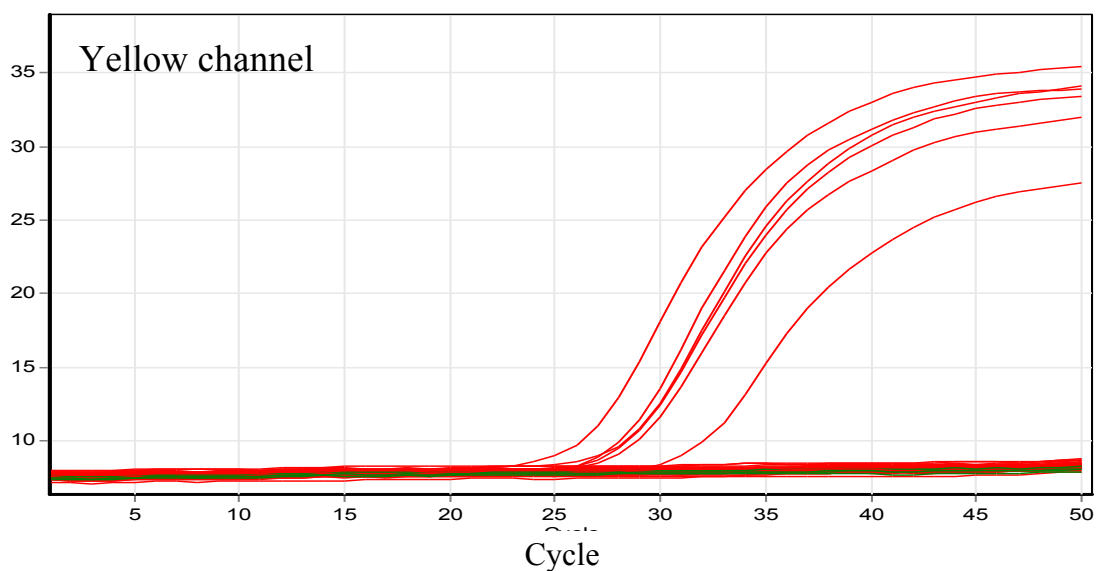


**Figure 2.10** Amplification of *tcdB* for samples 1-45 by multiplex real-time PCR. Red curves refer to *tcdB* positive samples while the green lines were *tcdB* negative samples.

Six samples positive for *tcdA* and *tcdB* were also positive for *cdtA* and *cdtB*. The mean Ct value for *cdtA* (Figure 2.11) was 29.66 (SD=2.5) which was higher than the mean Ct value of 27.66 (SD=1.6) for *cdtB* (Figure 2.12).



**Figure 2.11** Amplification of *cdtA* for samples 1-45 by multiplex real-time PCR. Red curves refer to *cdtA* positive samples while the red and green lines were *cdtA* negative samples.



**Figure 2.12** Amplification of *cdtB* for samples 1-45 by multiplex real-time PCR. Red curves refer to *cdtB* positive samples while the red and green lines were *cdtB* negative samples.

The genetic profile of 70 stool samples tested by multiplex real-time PCR that also had a cytotoxicity reported is shown in Table 2.6.

**Table 2.6** PCR and CTA result of the 70 samples tested by multiplex real-time PCR assay using stool DNA

CTA	<i>tcdA/tcdB</i>	<i>cdtA/cdtB</i>	No. of sample found
+	+	+	5 (7.1%)
+	+	-	27 (38.6%)
+	-	-	1 (1.4%)
-	-	-	19 (27.1%)
-	+	+	1 (1.4%)
-	+	-	17 (24.3%)
			Total 70

PCR for *tcdA/tcdB* was in agreement with stool CTA in 72.8% of samples. In one sample cytotoxicity of stool was reported positive, yet the *tcdA/tcdB* results were negative. 25.7% of isolates were positive for *tcdA/tcdB* but were reported negative in cytotoxicity testing. 8.5% of the 70 isolates were binary toxin gene positive. Of the 70 stool samples growing *C. difficile*, 33 were positive in the CTA (Table 2.6). The multiplex real-time PCR assay agreed with 32 of these samples and the one negative was also negative by conventional PCR of both stool and isolate DNA. The real-time PCR identified a further 18 faecal samples that were *tcdA* and *tcdB* positive of which all were confirmed positive by conventional PCR.

### 2.3.5 Validation of multiplex real-time PCR assay with faeces from patients suspected of CDI

To evaluate the applicability of multiplex real-time PCR for detection of toxigenic *C. difficile* in stool, 70 stool samples tested for *tcdA*, *tcdB* and binary toxin (*cdtA* and *cdtB*) by conventional PCR assay were compared to the results obtained by DNA extracted directly from the same stool sample by the automated system (QIAAsymphony). The results showed that the 42 samples (Table 2.7) detected positive by multiplex real-time PCR were also

positive by conventional PCR using stool DNA and 20 isolates were negative by multiplex real-time PCR were also confirmed negative by conventional PCR using stool DNA (Table 2.7). The multiplex real-time PCR was positive in eight additional samples and no false negatives were detected for stool testing by real-time PCR. These eight samples were previously reported as negative for toxin genes when DNA was extracted manually from stool.

**Table 2.7** Comparison of multiplex real-time PCR with conventional PCR (stool DNA)

Assay	No. of samples included	Results	Conventional PCR stool DNA		Sensitivity (%)	Specificity (%)
			Positive	Negative		
Multiplex RT-PCR (stool DNA)	70	Positive	42	8	100%	71.4%
		Negative	0	20		

The overall agreements of the multiplex real-time PCR and the conventional PCR using stool DNA were 100% sensitivity and 71.4% specificity (Table 2.7).

The result of real-time PCR testing of stool was compared to conventional PCR testing of isolate DNA (Table 2.8). Using the conventional PCR (isolate DNA) as a gold standard the sensitivity and specificity were 100% and 80% respectively.

**Table 2.8** Comparison of multiplex real-time PCR with conventional PCR (isolate DNA)

Assay	No. of samples included	Results	Conventional PCR isolate DNA		Sensitivity (%)	Specificity (%)
			Positive	Negative		
Multiplex RT-PCR (stool DNA)	70	Positive	45	5	100%	80%
		Negative	0	20		

From Table 2.8, five stool samples gave positive toxin PCR results but the corresponding isolates from the same stool were not found to be PCR-toxin gene positive.

### 2.3.6 Sensitivity and specificity of multiplex real-time PCR assay

The multiplex real-time PCR assay was more sensitive than conventional PCR in comparison with the gold standard method of CTA (Table 2.9).

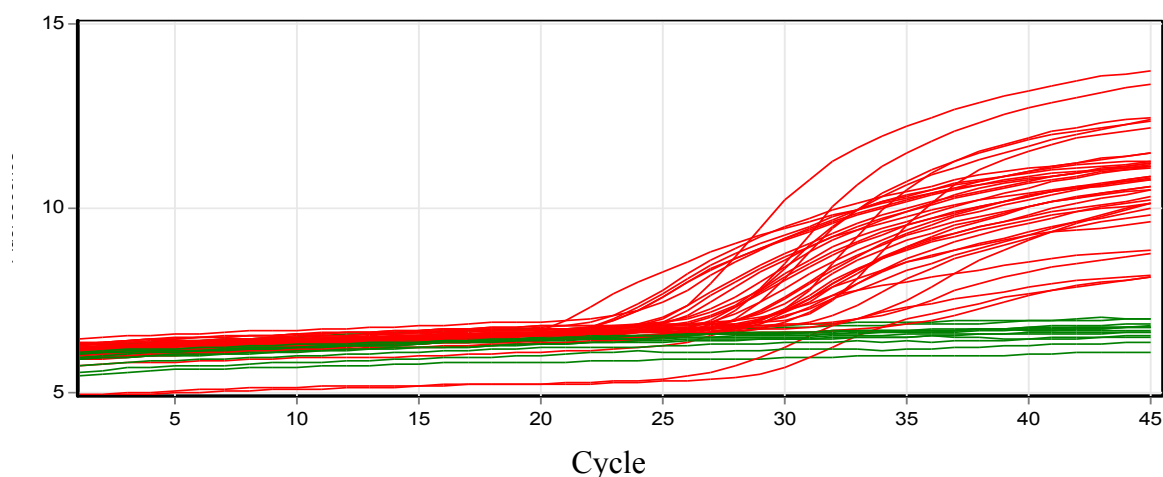
**Table 2.9** Comparison of two conventional PCR and multiplex real-time PCR with gold standard CTA

Assay	No. of samples included	Results	CTA Assay(no.)		Sensitivity (%)	Specificity (%)
			Positive	Negative		
PCR (isolate DNA)	70	Positive	29	16	87.8%	56.8%
		Negative	4	21		
PCR (stool DNA)	70	Positive	28	11	84.8%	70.2%
		Negative	5	26		
Multiplex RT-PCR (stool DNA)	70	Positive	32	18	97%	51.4%
		Negative	1	19		

DNA extracted from stool using manual kit was used for conventional PCR detection of toxin genes in stool. DNA extracted from stool using the robotic platform was able to detect all the positive stool samples found by manual DNA extraction. An additional eight out of 50 (16%) samples were found to be positive with robotic extraction of DNA.

### 2.3.7 Deletion assay (delta 117 detection)

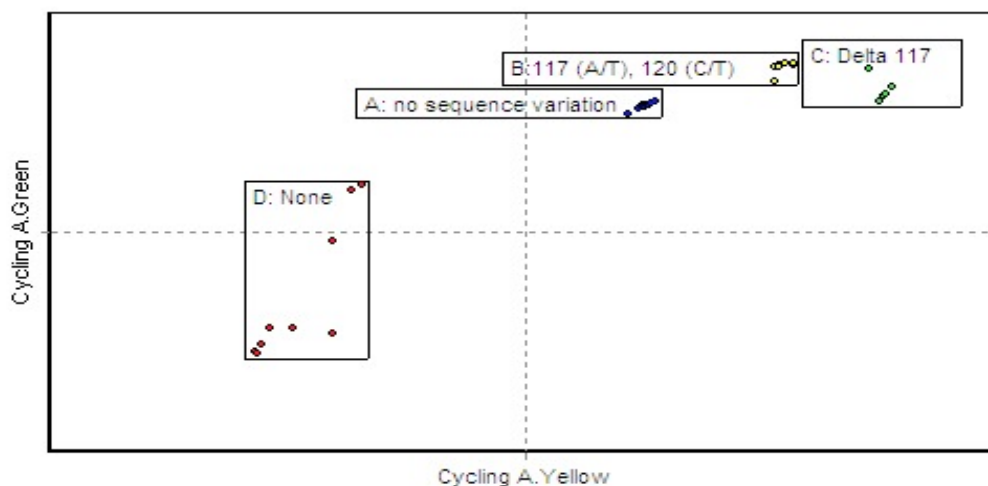
A PCR assay for the  $\Delta 117$  mutation (de Boer et al., 2010) was performed on all isolates and stool samples DNA using the three DNA extraction methods described previously. The real-time PCR for *tcdC* gave a mean value of 26.9 (SD=2.9) (Figure 2.13) which was comparable to the Ct value obtained by de Boer et al., (2010).



**Figure 2.13** Amplification of *tcdC* for samples 1-45 by multiplex real-time PCR. Red curves were *tcdC* positive samples while the green lines were *tcdC* negative samples.

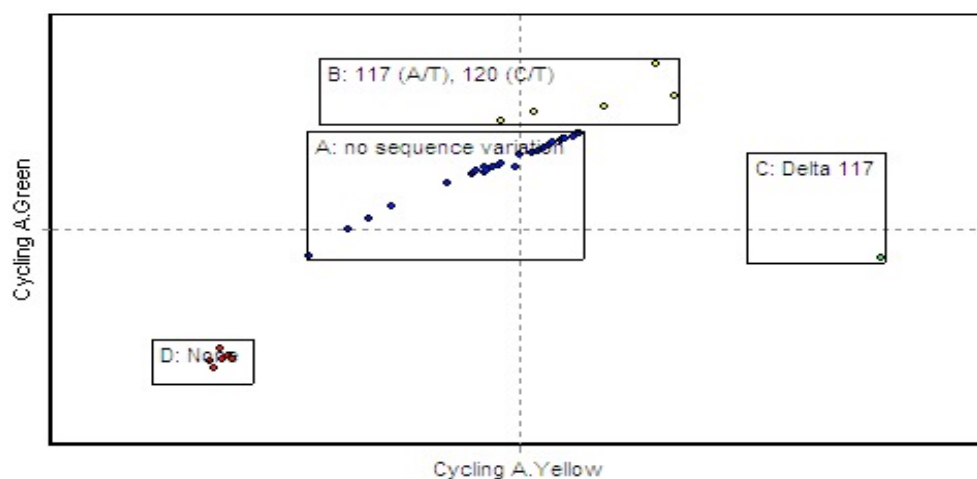
The real-time PCR data was imported to scatter plotting software which showed four clusters (A, B, C and D, Figure 2.14). In Figure 2.14, DNA was extracted from pure culture. The wild-type probe was detected on the green channel whereas the mutant probe was on the yellow channel. As expected the strongest reading for the yellow channel was the control isolates for  $\Delta 117$  (Figure 2.14, cluster C). Isolates that had the predicted wild-type *tcdC* genotype were clustered together (A). Those with some base pair variation clustered in B and the control isolates for  $\Delta 117$  and one clinical isolate clustered together in C. Isolates where no *tcdC* gene product was produced clustered together as group D.





**Figure 2.14** Delta 117 assay used DNA extracted from pure culture. A: no sequence variation, B: 117 (A/C) and 120 (C/T), C: Delta 117, D: no *tcdC* detected. X axis signal from mutant probe, Y axis signal from wild-type probe.

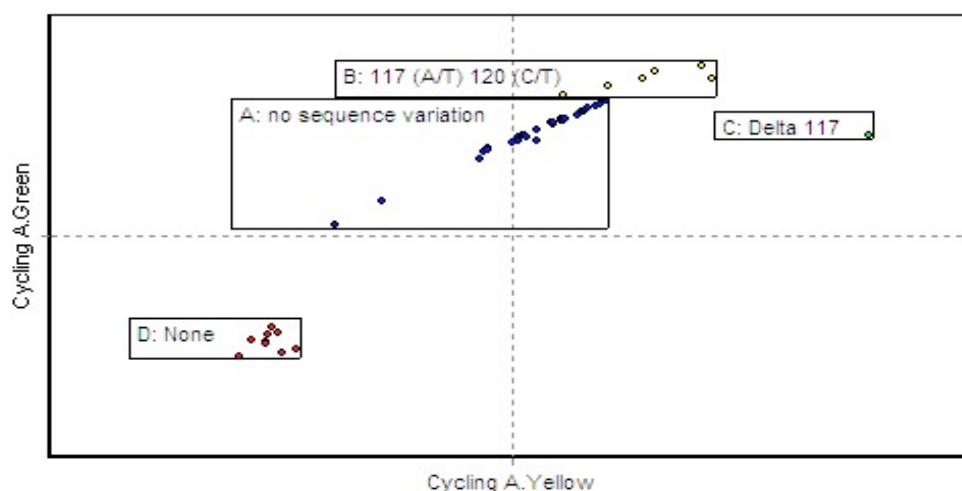
To see if similar clusters were obtained by direct testing from stool, scatter plotting software was used to analyse the real-time PCR data obtained from stool DNA using a manual kit. Similar clusters were obtained to Figure 2.14 and are shown in Figure 2.15.



**Figure 2.15** Delta 117 assay used DNA extracted directly from stool samples using QIAamp® DNA Stool Mini Kit A: no sequence variation, B: 117 (A/C) and 120 (C/T), C: stool sample 12 confirmed as  $\Delta$ 117, D: no *tcdC* detected.

To see if an automated robotic platform performed as well as manual extraction of DNA, scatter plotting software was used to analyse the real-time PCR data obtained from stool

DNA using the robotic extraction method. Similar clusters were obtained to Figure 2.15 and are shown in Figure 2.16.



**Figure 2.16** Delta 117 assay used DNA extracted directly from stool samples using QIA Symphony SP automated systems A: no sequence variation, B: 117 (A/C) and 120 (C/T), C: stool sample 12 confirmed as  $\Delta$ 117, D: no *tcdC* detected.

The  $\Delta$ 117 PCR assay detected the *tcdC*  $\Delta$ 117 mutation in 1 of 70 samples (Cluster C, Figure 2.14, 2.15 and 2.16) and all four positive control isolates (Figure 2.14). The presence of hypervirulent *C. difficile* in one of the samples tested was confirmed by PCR-ribotyping of the isolate (Chapter 3) which showed an identical ribotyping pattern to the control 027 strains. Sequencing of the *tcdC* PCR product confirmed the presence of the  $\Delta$ 117 mutation consistent with 027 strains (Figure 2.5, isolate 12). Sequencing of isolates clustering in groups A, B and C show why they cluster in different groups. As expected, the strongest ratio for the green/yellow channel was obtained by isolates most similar to the wild-type. Isolates differing by one base pair from the wild-type gave stronger signals with the yellow channel and isolates matching the wild-type gave the weaker yellow channel signals. Isolates without *tcdC* gave weak signals with both probes and were in a separate cluster.

## 2.4 Discussion

PCR testing of toxin genes for the detection of *C. difficile* is recognised as rapid, sensitive and specific, however is yet to be widely used as a standalone test (Cohen et al., 2010). In the current study, conventional PCR using DNA extracted directly from stool samples gave a sensitivity of 82% and specificity of 80% when compared to conventional PCR using DNA extracted from pure culture. When DNA was extracted from stool using the robotic system, all samples detected as positive using the manual extraction method were found to be positive with an additional eight samples being positive. These corresponding eight samples detected negative by conventional PCR using stool DNA when compared to conventional PCR using isolate DNA.

It would be expected that stool extraction would have a lower sensitivity compared to isolate DNA as there would be inhibitors present in stool. The fact that the robotic system identified eight samples more than the manual kit would seem to indicate that this system of DNA extraction was able to either overcome some of the inhibitors present in stool or extract DNA of better quality for testing. This finding was supported by the high sensitivity (100%) of multiplex real time PCR using robotic DNA when compared to conventional PCR. This supports the conclusion that real-time PCR performs as well as conventional PCR for testing of stool DNA.

Five samples testing *tcdA* and *tcdB* positive by PCR using DNA extracted directly from stool samples by two DNA extraction methods (manual kit and QIA Symphony automated system) were not found to have isolates that were toxin positive. This may be explained if different types of *C. difficile* strains are present in the same stool sample. This finding has been reported by Wroblewski et al. (2009) when they showed that more than one strain can be present in a given stool sample.

The sensitivity of multiplex real-time PCR when compared to CTA was 97% (Table 2.9). This result was similar to that reported by de Boer et al. (2010) who showed a sensitivity of 100% for the diagnosis of CDI using CTA as the “gold standard”. The high sensitivity obtained for the multiplex real-time PCR may be related to the use of the QIAasympyphony SP automated system for extraction of DNA directly from stool samples. In a study published by Stamper et al. (2009b) using anaerobic toxigenic bacterial culture, as the “gold standard,” and DNA extracted by automated system (NucliSENS easyMAG platform) the sensitivity and specificity for ProGastro Cd assay (which is a new commercial TaqMan PCR assay that detects *tcdB*) were 77.3% and 99.2% respectively and for Wampole TOX-B test (a cell culture cytotoxicity neutralization assay (CCCNA)) were 63.6% and 99.2% respectively (Stamper et al., 2009b). The conclusion was that real-time PCR using DNA extracted by an automated system was superior to CTA for diagnosis of *C. difficile* infection.

Detection of *tcdA* and *tcdB* by multiplex real-time PCR using our modified primers and probes derived from Wroblewski et al. (2009) showed that the *tcdB* had a higher Ct value than *tcdA*. Wroblewski et al. did not report the Ct values obtained in their assay. To improve the Ct values for *tcdB* it might be possible to revisit the design of the *tcdB* primers and probes which may lead to improved performance of the *tcdB* real-time assay. Despite the high sensitivity of the multiplex real-time PCR, the test showed a specificity of (51%) when using CTA as a gold standard. This is most likely due to the large number of ‘false’ positives (18 out of 70 samples, Table 2.9). The determination of false positives is reliant on how good the ‘Gold standard’ is and it has been shown that the CTA test has poor sensitivity compared to CTA determined by cytotoxic culture technique (Stamper et al., 2009b). A similar study stated that using CTA testing was unreliable for detection of *C*

*difficile* (Eastwood et al., 2009). As the purpose of the current study was to validate that real-time PCR performed as well as conventional methods and that the robotic system was able to identify positive stool samples, future validation studies should be compared to the better gold-standard of cytotoxic culture.

Although the contribution of binary toxin genes to the virulence of *C. difficile* strains causing infections is still poorly understood, the highly related binary toxin in *Clostridium spiroforme* has been implicated as a virulence factor as it has been shown to be involved in the pathogenesis of intestinal disease in rabbits (Geric et al., 2003). The prevalence of binary toxin-positive, A<sup>-</sup> B<sup>-</sup> strains has been reported at 2.0% (8 of 402) in one study (Geric et al., 2003). In our study we did not isolate any binary toxin positive *tcdA-tcdB*- strains, however since these strains do occur it would seem important to include testing for *cdtA/cdtB* genes.

TaqMan based real-time PCR using the three different methods of DNA extraction identified the *tcdC* Δ117 mutation. However, the real-time PCR using DNA extracted by the automated system detected the *tcdC* Δ117 mutation in a shorter time compared to the other two extraction methods. This study is the first to use scatter plotting software to analyse Δ117 real-time PCR results. The use of scatter plotting software was able to identify an isolate and corresponding stool sample containing the epidemic 027/NAP1 strain. In Figure 2.14, the control strains and isolate 12 cluster together with the strongest yellow channel signal. However the wild-type isolates with single base transition also cluster close by (Cluster B, Figure 2.14). Pure wild-type strains with *tcdC* also clustered with strong signals for both the green and yellow channels. The strongest signal for the mutant probe (yellow channel) was obtained in stool samples for the sample shown to contain isolate 12. To confirm the usefulness of the Rotor-gene scatter plotting software to

identify stools containing 027 ribotype strains, more clinical isolates would be need to be investigated. Our study support the results obtained by de Boer et al. (2010), that a TaqMan based real-time PCR assay is able to detect the *tcdC*  $\Delta$ 117 mutation without the need for melting curve analysis (de Boer et al. 2010). Testing for  $\Delta$ 117 on all samples positive for *tcdA*, *tcdB*, *cdtA* and *cdtB* would seem to be an effective way to identify patients with 027 infection and help minimise the spread of the epidemic strain in hospitals and healthcare facilities.

In conclusion multiplex real time PCR for detection of *C. difficile* toxin genes *tcdA* and *tcdB* as well as binary toxin *cdtA* and *cdtB* using DNA extracted by an automated system has been shown to give reliable results when compared to CTA and conventional PCR. Including binary toxin testing would seem to be important especially as *tcdA*- *tcdB*- binary toxin positive isolates have been reported. Furthermore, the  $\Delta$ 117 PCR may provide valuable information for quickly identifying 027 isolates so that the spread of epidemic *C. difficile* PCR ribotype 027 may be brought under control.

## **CHAPTER 3**

### **Development of an RT-PCR ribotyping technique**

### 3.1 Introduction

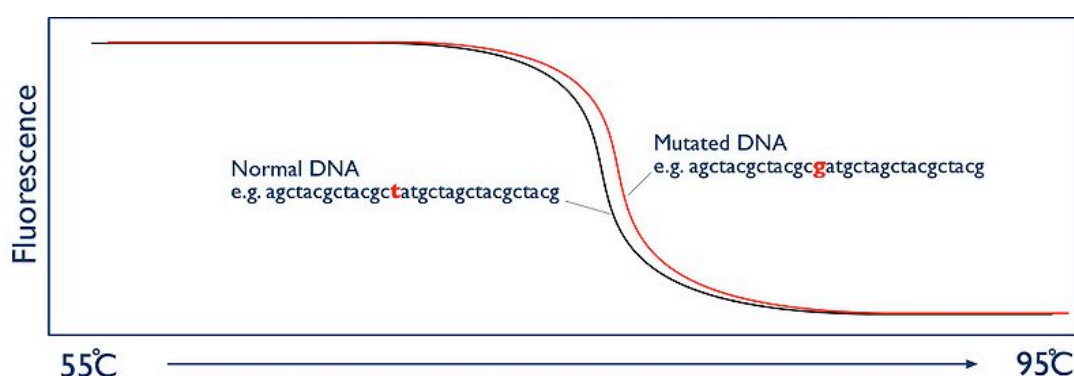
The methods used to type *Clostridium difficile* have been reviewed in Chapter one. The main methods used around the world are REA of the total bacterial genome (Kato et al., 1993, Kuijper et al., 1987), PFGE, AP-PCR (Fawley and Wilcox, 2002, Killgore and Kato, 1994, Rodriguez-Palacios et al., 2007), and PCR-ribotyping developed by Kostman et al. (1992). PCR-ribotyping was adapted by Gurtler (1993) for typing of *C. difficile*. PCR-ribotyping produces multiple PCR products due to the presence of several alleles of the rRNA operon differing by the length of the ISR located between the 16S and the 23S rRNA genes.

The single most important epidemic strain causing CDAD in North America and Europe types as BI by REA, North American (NA) pulsed-field type 1 (NAP1) by PFGE, and 027 by PCR-ribotyping (BI/NAP1/027) (Kuijper et al., 2006, McDonald et al., 2005, Pepin et al., 2004). Restriction endonuclease analysis of the total bacterial genome is highly discriminatory but is still a crude method. PFGE may require up to 6 days to complete. PCR-ribotyping has been reported to provide a discriminatory, reproducible, and a simple alternative to other typing methods (Cartwright et al., 1995). This technique has a number of advantages over other methods; specifically, PCR-ribotyping has been shown to be more discriminatory than arbitrarily primed PCR (Collier et al., 1996), and is quicker and simpler than PFGE. PCR-ribotyping also avoids the excessive endogenous nuclease activity of some *C. difficile* isolates that renders them untypeable by PFGE (Stubbs et al., 1999). The emergence of the hypervirulent strain has increased the need for rapid typing of *C. difficile*. Despite the advantages of the PCR-ribotyping method it still requires analysis of electrophoretically separated bands and analysis using GelCompar software. Faster typing techniques will enable the quicker detection of hypervirulent strains and the timely implementation of infection control strategies.



A new technique known as high resolution melt (HRM) analysis can be used to characterize DNA samples according to their dissociation behaviour as they transition from double stranded DNA (dsDNA) to single stranded DNA (ssDNA). The melt characteristics of PCR products depends on GC content, length, sequence and heterozygosity and is best monitored with saturating dyes that fluoresce in the presence of double-stranded DNA with increasing temperature (Reed et al., 2007). Real-time PCR instruments that are equipped to perform HRM do so by acquiring data at small temperature increments such as 0.02°C. This enables the melt curve to be resolved with much greater power. HRM analysis can be used to detect single base sequence variations such as SNPs (single nucleotide polymorphisms) (Figure 3.1) or to discover unknown genetic mutations. It may be possible to analyse the pattern of different alleles produced by PCR-ribotyping through HRM analysis.

The aim of this chapter is to develop a *C. difficile* real-time PCR typing method based on the HRM analysis of PCR products obtained from the ISR of *C. difficile*. This technique will then be compared to ribotyping for its discriminatory power.



**Figure 3.1** DNA Melt Curve for High Resolution Melting Analysis.

The research questions we aimed to answer in this part of the study were:

- 1- Can HRM analysis be used to identify clusters of related isolates including PCR ribotype 027?
- 2- Can the development of a RT-PCR-ribotyping technique lead to a quicker and cost effective screening of epidemic strains?
- 3- Can HRM PCR-ribotyping be performed directly from stool?

## **3.2 Materials and Methods**

### **3.2.1 Bacterial isolates**

A total of 93 *C. difficile* isolates were collected as detailed in section 2.2.1. Five control strains were used as outlined in 2.2.1.

### **3.2.2 DNA extraction**

The same DNA extracted from isolates and stool samples as detailed in sections 2.2.2 and 2.2.3 was used in this study.

### **3.2.3. Conventional PCR-ribotyping (ISR amplification)**

All isolates were typed using the PCR-ribotyping method described by Bidet et al. (1999). Briefly, amplification reactions were performed in 50 µL reaction volumes, composed of 5 µL of buffer 2 (PE), 2.5 mM MgCl<sub>2</sub>, 16 µM dNTPs, 0.5 µM of both forward and reverse primers, 2U of AmpliTaq Gold (Perkin Elmer, Cetus, USA) and 5 µL of the genomic template DNA or 5 µL sterile water as a negative control. Amplifications were carried out in a GeneAmp PCR system 2700 for 1 cycle of 10 minutes at 94°C; 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; followed by a final extension cycle of 10 min at 72°C. Amplification products were fractionated by electrophoresis using polyacrylamide gel (Bio-Rad, Hercules, USA) 15% TBE (Tris-borate- EDTA) buffer (Eurobio, Les Ulis, France) to give more resolution of banding patterns. Ten µl of PCR product from each PCR reaction was then mixed with 2.5 µl loading buffer and loaded sequentially into the wells. Marker VI DNA (Roche, Indianapolis, USA) was used in the first and last wells. A constant 200 volt (6.0V/cm) charge was applied across the gel for 90 min in 0.5 X TBE buffer and analysed under 280 nm ultraviolet (UV) light after staining for 20 min with 0.5 µg/µl ethidium bromide. Gel images were acquired by Fluor-S<sup>tm</sup> MultiImager (Bio-Rad) and

saved as high-resolution TIFF files. Gel images were saved as TIFF files and transferred to GelCompar II software V4.0 (Applied Maths, Austin, USA) to analyse band patterns. Each gel was standardised using a band tolerance of 0.5%. Cluster analysis was performed using Pearson coefficient with arithmetic (UPGMA) mean.

### **3.2.4 Real-time PCR with HRM analysis**

Real-time PCR and HRM were performed in duplicate in a Rotor-Gene 6000 (QIAGEN). As good reproducibility was obtained with duplicates, all isolates were measured from then on singly to obtain all HRM profiles in a single run (maximum capacity for a single run is 100 samples). The primer sequences used for the real-time PCR assay were the same primers as used in the conventional PCR ribotyping. All PCR mixtures (50  $\mu$ L) contained 5  $\mu$ L of buffer 2 (PE), 2.5 mM  $MgCl_2$ , 16  $\mu$ M dNTPs, 0.5  $\mu$ M of both forward and reverse primers, 2U of AmpliTaq Gold (Perkin Elmer), 1.5  $\mu$ L of 1 X SYTO-9 intercalating dye (Invitrogen) and 5  $\mu$ L of the genomic template DNA (or sterile water as a negative control). The thermocycling consisted of an initial incubation at 50°C for 2 min and 1 cycle of 95°C for 10 min, followed by 45 cycles of 95°C for 5 s and 60°C for 60 s, with data acquired on the 60°C step in the green channel. The PCR HRM analysis was performed by heating the amplicon DNA gradually from 80°C up to 90°C in 0.1 increments in order to generate a melt curve. Raw melting-curve data was normalized for the 83 isolate and 5 reference controls by setting the pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all samples to uniform values. Pre-melt signals were uniformly set to a relative value of 100%, while post-melt signals were set to a relative value of 0%. The second step was to determine a temperature along the x axis of the normalized melting curves at which point the entire double-stranded DNA is completely denatured. The third step was to further analyze the differences in melting-curve shapes by subtracting the curves from a reference curve (also called the 'base curve'), thus generating a difference plot curve, which helps to

cluster samples into groups that have similar melting curves (i.e., those with the same genotype). The final step was to generate the difference graph using the curve from ribotype 027 R20291 reference strain to analyse how close or different the isolates were to the ribotype 027 strain.

### **3.2.5 Real-time PCR with HRM analysis using DNA extracted from stool**

Real-time PCR with HRM was repeated as described in section 3.2.4 using DNA extracted directly from 21 stool samples by QIAamp® DNA Stool Mini Kit (QIAGEN) to check if similar clusters could be identified directly in stool samples.

### **3.2.6 *ScreenClust* analysis**

Clustering analysis was performed with a prototype version of the *ScreenClust* software (QIAGEN, Hilden, Germany). Raw HRM data from the Rotor-Gene operating software was imported into *ScreenClust* software. To determine differences between individual samples, normalized melt curves are first differentiated in *ScreenClust*. The HRM curves are derived by selecting two normalized regions, the first occurring prior to the melting of the double-stranded product and the second following complete separation of the two strands. Each region is generated by default by the software but may be manipulated manually to achieve optimum results. Default settings were used for the analysis. These regions function to normalize the fluorescence of the melt curves from the raw channels by averaging all starting and ending fluorescence values such that the end point value of each sample is identical to the average. This allows for the melting curve profile of each isolate to be analyzed relative to those of the others. Following this, a residual plot is generated by subtracting all the differentiated curves by the composite median of all curves. The residual plot is used as the data basis to form the principal component analysis that extracts a range of characteristics for each curve. The software enables a supervised mode which is used for

classification of unknown samples into known groups using known samples as controls and unsupervised mode is used to find de novo data groups when there is no prior knowledge on the number and kind of genotypes present in the data set. The classification of clusters of isolates was performed in unsupervised mode as no controls for each individual clusters were available. After the initial analysis, a cluster of 12 isolates contains the 027 strains was present, so the data was then analysed in supervised mode using the R20291 ribotype 027 strain as a control. No template controls (NTC) and samples showing Ct values greater than 25 on real-time PCR were removed from the analysis as a lack of melt curve features from weak PCR products affects the normalization. A Ct value of 25 was selected as isolates with Ct values above 25 were also found to have correspondingly weak ribotyping bands produced that did not enable them to be accurately ribotyped.

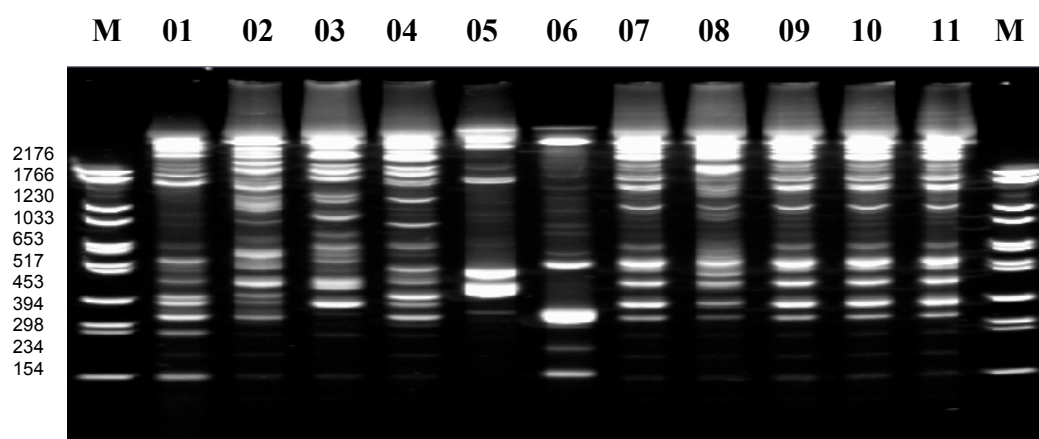
### **3.2.7 Discriminatory power measurement**

The discriminatory power of a typing method is its ability to distinguish between unrelated strains. Simpson's index of diversity was used based on the probability that two unrelated strains sampled from the test population will be placed into different typing groups (Hunter and Gaston, 1988). To assess the ability of HRM using *ScreenClust* software for data analysis to type isolates the discriminatory power was calculated by Simpson's index of diversity (Hunter and Gaston, 1988) and compared to the discriminatory power of conventional PCR-ribotyping of the same groups of isolates.

### 3.3 Results

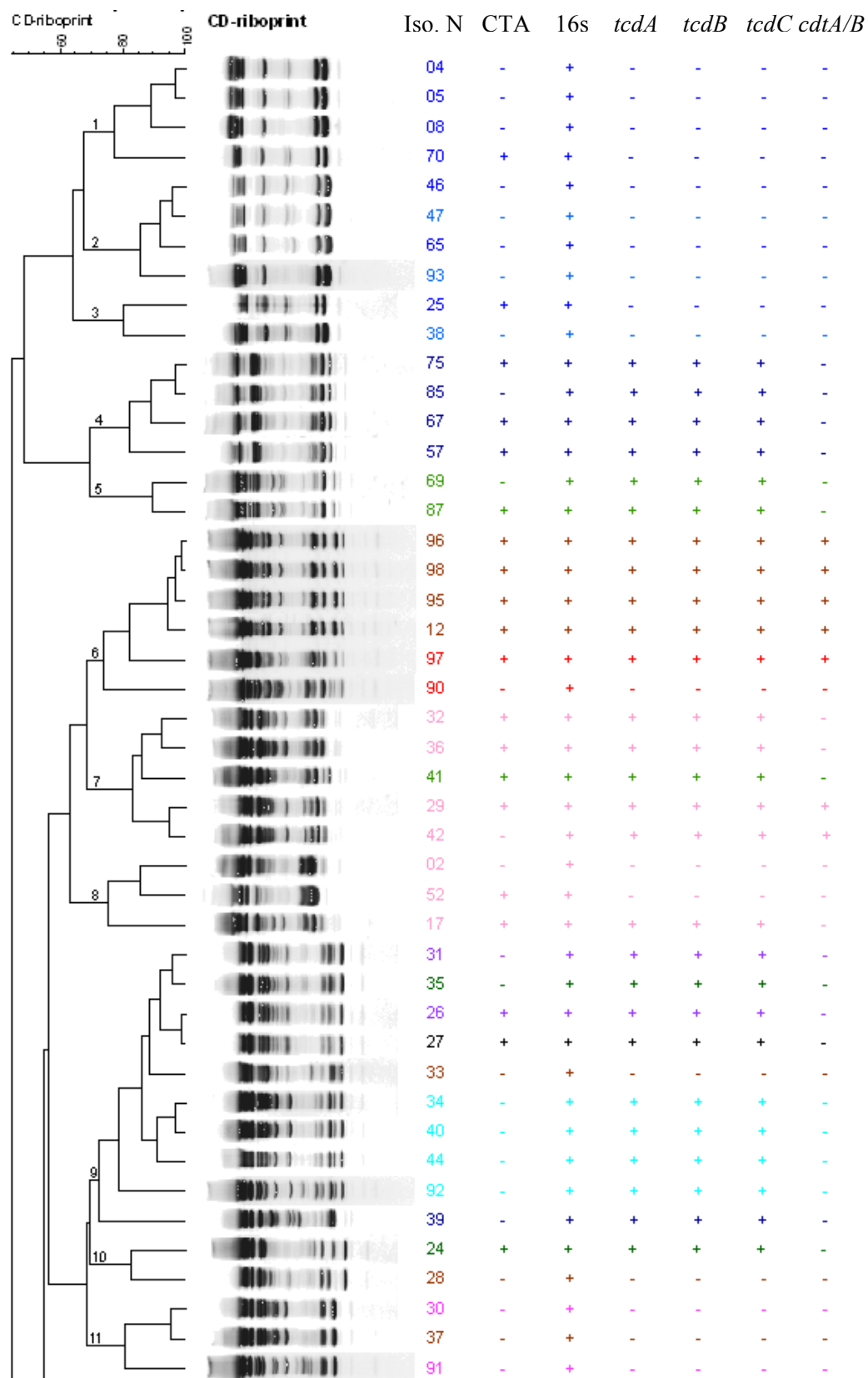
#### 3.3.1 PCR ribotyping

A total of 93 *C. difficile* clinical isolates and five reference strains (ATCC 9689 and four *C. difficile* ribotype 027 strains) were analysed using PCR ribotyping. The PCR-ribotyping patterns of seven clinical isolates and four ribotype 027 reference strains (KI, R20291, M7404 and CD196) are shown in Figure 3.2. The results of remaining isolates are shown in Figure 3.3. Control isolate M7404 from the Canadian outbreak shows a different banding pattern but is still sufficiently similar to the other 027 control strains to cluster with them after UPGMA analysis (Figure 3.3).



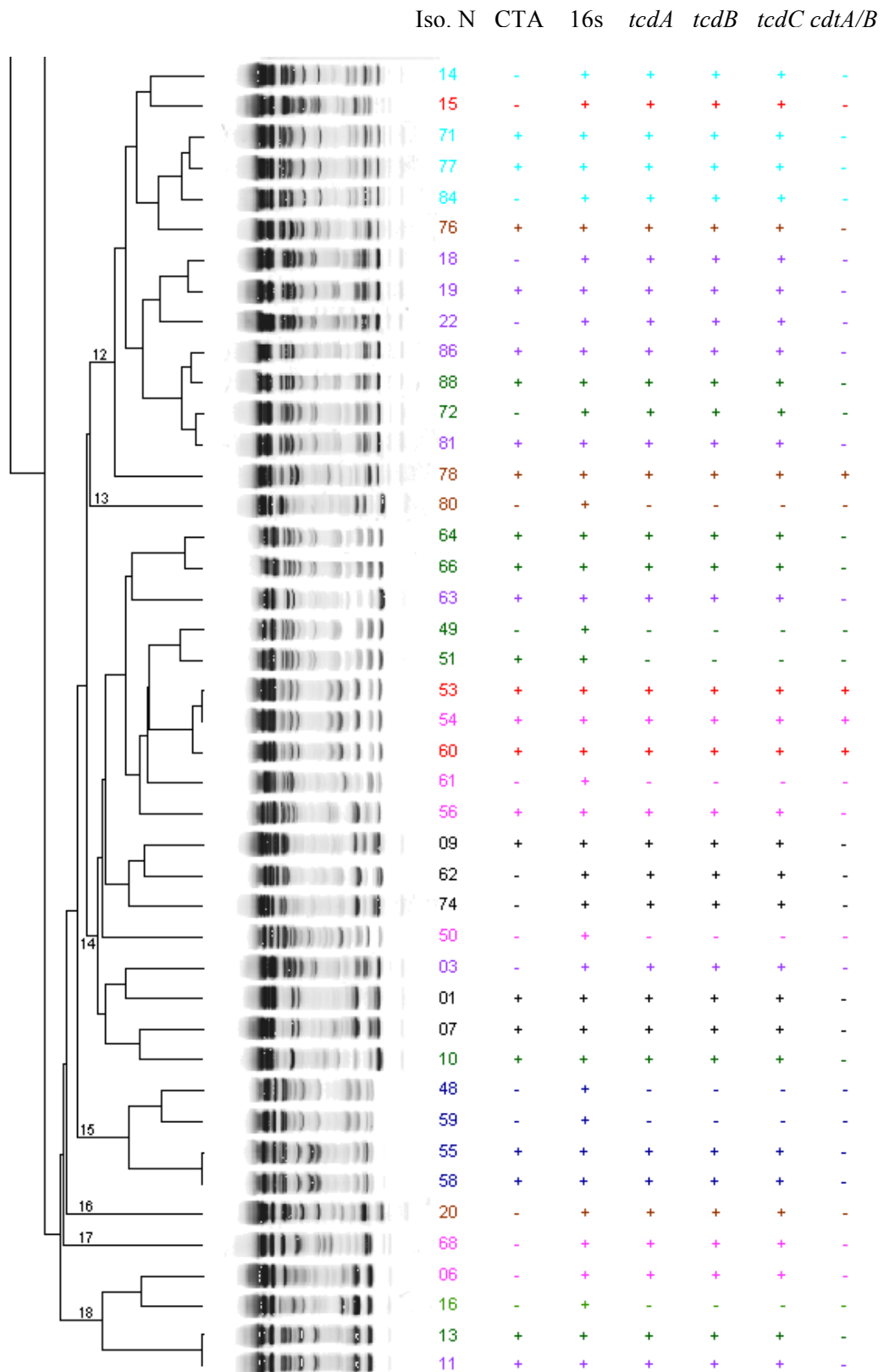
**Figure 3.2** Representative PCR ribotype profiles from *C. difficile*; lanes 01-06 representing *C. difficile* isolates number 89-94; lanes 07-10 *C. difficile* ribotype 027 controls 95, 97, 96 and 98; (KI, M7404 R20291 and CD196) respectively. Lane 11 *C. difficile* clinical isolate number 12 suspected as 027 ribotype strain. M indicates molecular marker VI (154-2176 bp).

Clinical isolate numbers 21, 23, 43, 45, 73, 79, 82, 83, 89 and 94 produced weak ribotyping patterns on polyacrylamide gel electrophoresis and Ct values greater than 25 on real-time PCR, so these isolates were excluded from GelCompar II analysis. The diversity of 83 *C. difficile* clinical isolates from patients at the Austin Hospital is illustrated by the UPGMA dendrogram (Figure 3.3). After considering the similarity in banding patterns 18 groups were found at 70% homogeneity using Person coefficient with UPGMA mean (Figure 3.3).



**Figure 3.3** *C. difficile* ribotyping UPGMA dendrogram showing the ribotype patterns obtained by classic polyacrylamide gelelectrophoresis (dendrogram continued on next page). Coloration of isolate numbers relates to cluster groups outlined in section 3.3.3. The numbers in the dendrogram indicates the organism groups having a 70% similarity.



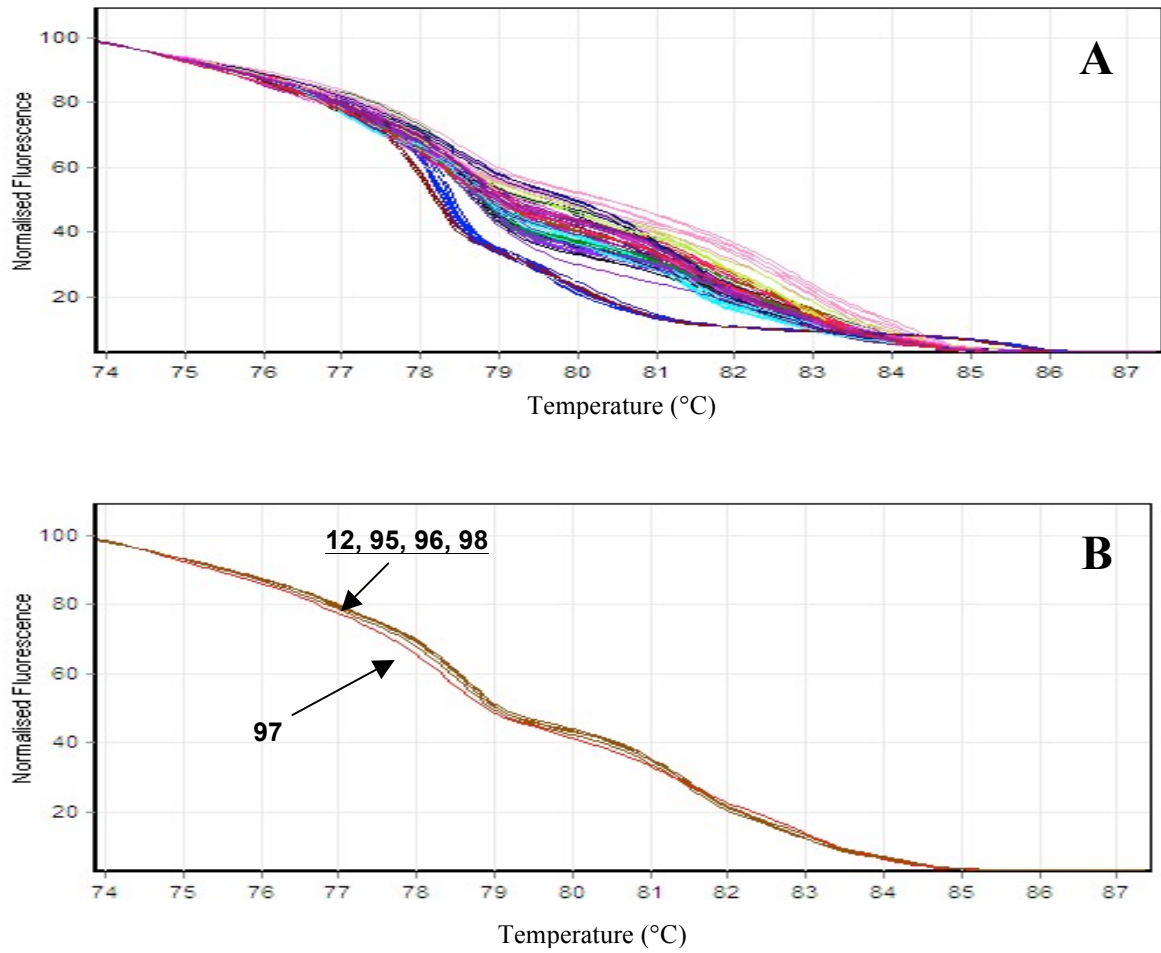


**Figure 3.3 Continued** *C. difficile* ribotyping UPGMA dendrogram showing the ribotype patterns obtained by classic polyacrylamide gel electrophoresis. Coloration of isolate numbers relates to cluster groups outlined in section 3.3.3. The numbers in the dendrogram indicates the organism groups having 70% similarity.

Four pairs of isolates 26 and 27, 53 and 54, 55 and 58, 96 and 98 showed 100% identity (Figure 3.3). Clinical isolate number 12 represented in lane number 11 of Figure 3.2 was identified as 027 ribotype because it showed 100% identity with the 027 ribotyping reference strains (KI, R20291 and CD196). The toxin profile listed in Figure 3.3 also shows it to have a profile consistent with 027 strains i.e. *tcdA*, *tcdB*, *cdtA*, *cdtB* and *tcdC* all positive.

### **3.3.2 HRM data analysis**

Real-time PCR and HRM analysis were performed in duplicate on a Rotor-Gene 6000 (QIAGEN). A total of 93 *C. difficile* clinical isolates and five reference strains (ATCC 9689 and four *C. difficile* ribotype 027 strains) had real-time PCR performed using the ribotyping primers. Clinical isolate numbers 21, 23, 43, 45, 73, 79, 82, 83, 89 and 94 produced Ct values greater than 25 for real-time PCR and were excluded from HRM analysis due to the weak melting profiles. These isolates also produced weak ribotyping patterns on polyacrylamide gel electrophoresis, as reported in section 3.3.1. HRM normalization was performed on the remaining 83 isolates and five reference strains and is shown in Figure 3.4.

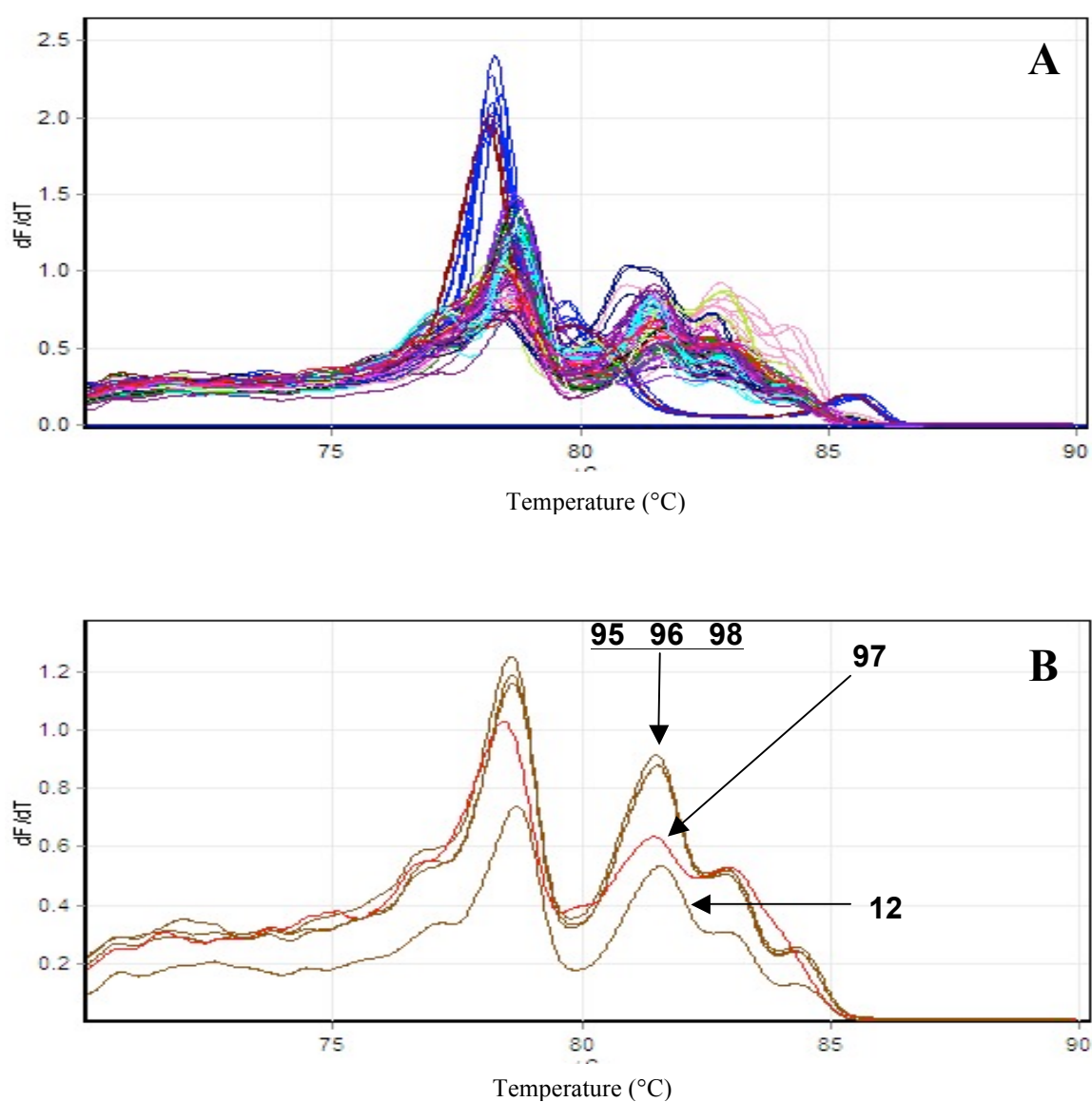


**Figure 3.4** HRM normalized graph for (A) 83 *C. difficile* clinical isolates and five control strains and (B) four ribotype 027 reference strains and *C. difficile* clinical isolate number 12. Isolate 97 (M7404) in (B) shows a slightly different melt curve consistent with the differences found in the ribotype pattern in Figure 3.3.

In Figure 3.4B it can be seen how closely clinical isolate number 12's melt curve corresponds with that of ribotype 027 strains R20291, KI, and the historical strain CD196 while the M7404 Canadian reference strain has a slightly different melt curve as shown in Figure 3.4B.

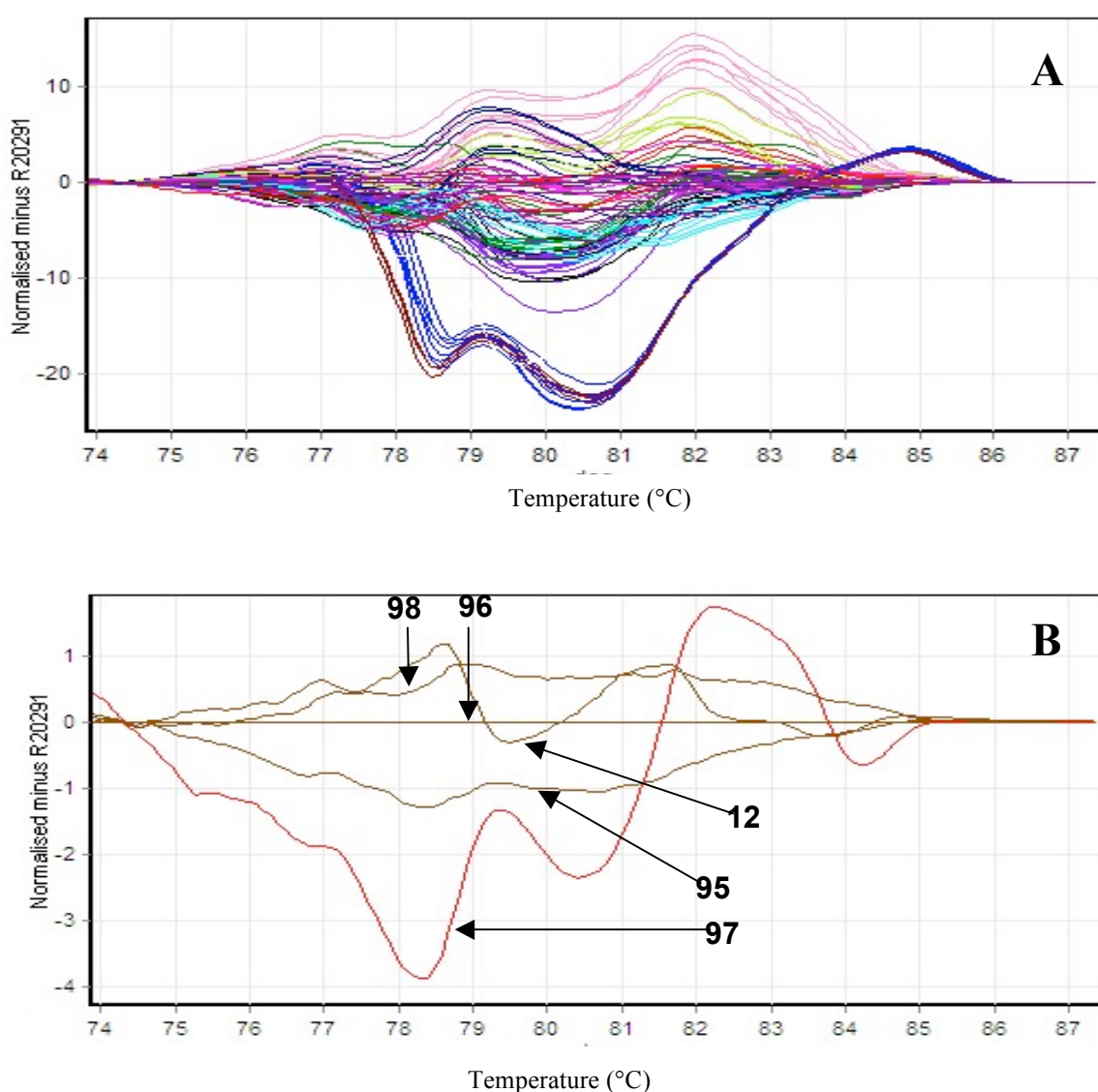
The melt peak curves for 83 isolates and five reference strains are shown in Figure 3.5A. In Figure 3.5B the melt peak curves are shown for the control strains and isolate 12. The signal strength is weaker for isolate 12, however the shape of the curve is very similar to that of the control strains except for control strain 97 (M7404). As expected from the melt

curve, M7404 shows a different peak curve to the other control isolates. The number of peaks present is to be expected as PCR-ribotyping produces a number of PCR products.



**Figure 3.5** HRM melt peak analysis for (A) 83 *C. difficile* clinical isolates and 5 control strain (B) four ribotype 027 reference strains and *C. difficile* clinical isolate number 12.

A difference curve was constructed by subtracting the melt curve variance at each temperature from the control strain R20291 (isolate 96). These differences curves for the 83 isolates are shown in Figure 3.6A and for the controls and isolate 12 in Figure 3.6B.



**Figure 3.6** Genotyping of *C. difficile* clinical isolates using high-resolution amplicon melting-curve analysis with differences plotting of 83 isolates and five controls (A). R20291 was the reference genotype (base curve) for the difference analysis. B difference plots for three *C. difficile* ribotype 027 numbered as 95, 97, and 98; (KI, M7404 and CD196) and clinical isolate number 12. Ribotype 027 R20291 number 96 was the reference genotype (base curve) for the HRM analysis (plots as a straight line at zero).

Figure 3.6A shows that the curves differ from that of the control 027 strain (isolate 96) from around +15 at 82°C to around -24 at 80.5°C. In Figure 3.6B it can be seen that the difference curves for the control strains (except M7404) are all within +/- 1 including isolate 12. Isolate 97 (control M7404) on the other hand has greater values of difference yet not as great as some other curves shown in Figure 3.6A.

Using R20291 ribotype 027 reference strain for HRM genotyping, Rotor-Gene software can calculate from these difference curve values the likelihood that isolates are related. KI, CD196 ribotype 027 control strains and isolate number 12 have confidences of 94.33%, 97.28% and 97.76% respectively of being the same genotype as R20291 (Table 3.1). The M7404 ribotype 027 Canadian strain showed a confidence of 71.36%, the lower confidence of R20291 is expected from the different pattern shown in Gelcompar II (Figure 3.3).

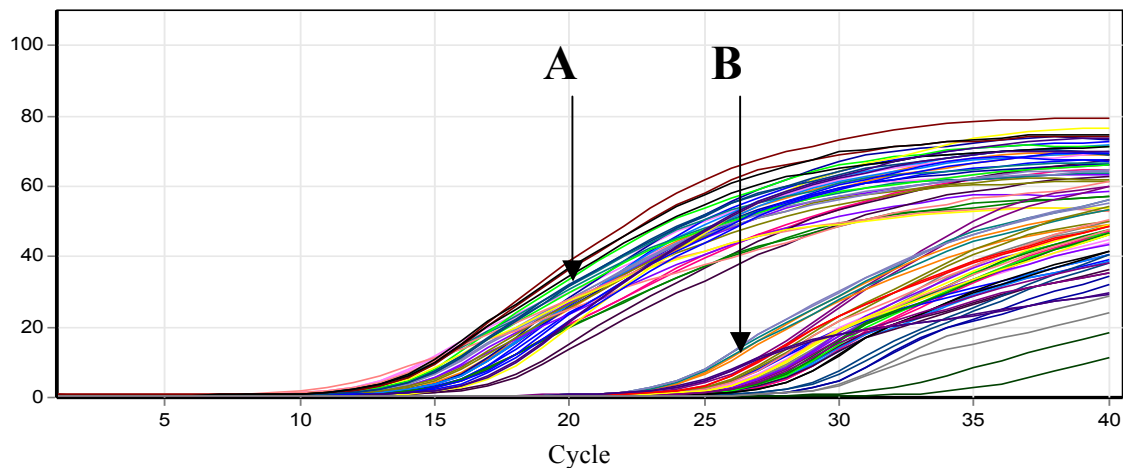
**Table 3.1** Confidences of KI, M7404 and CD196 and isolate number 12 to R20291

Seq. Number	Name	Genotype	Confidence %
95	KI	R20291	94.33
96	R20291	R20291	100.00
97	M7404	Variation	71.36
98	CD196	R20291	97.28
12	Clinical isolate	R20291	97.76

### 3.3.3 Real-time PCR with HRM analysis using DNA extracted from stool

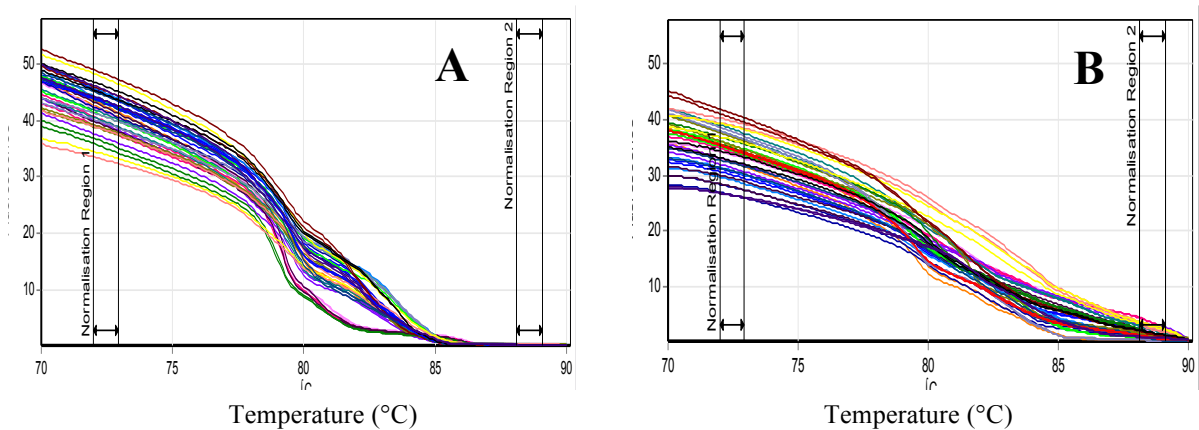
The real-time PCR with HRM analysis was performed on the Rotor-Gene 6000 (QIAGEN) in duplicates using DNA extracted from pure culture of 20 *C. difficile* clinical isolates and DNA extracted from stool samples of these 20 isolates to be cycled in the same run. Figure 3.7 shows the amplification profile of real-time and the differences in Ct values between group A (isolate DNA) and group B (stool DNA).

The mean Ct value for group B (Figure 3.7) was 25.7 (SD = 1.4) which was higher than the Ct value for group A which was 13.2 (SD = 1) (Figure 3.7).



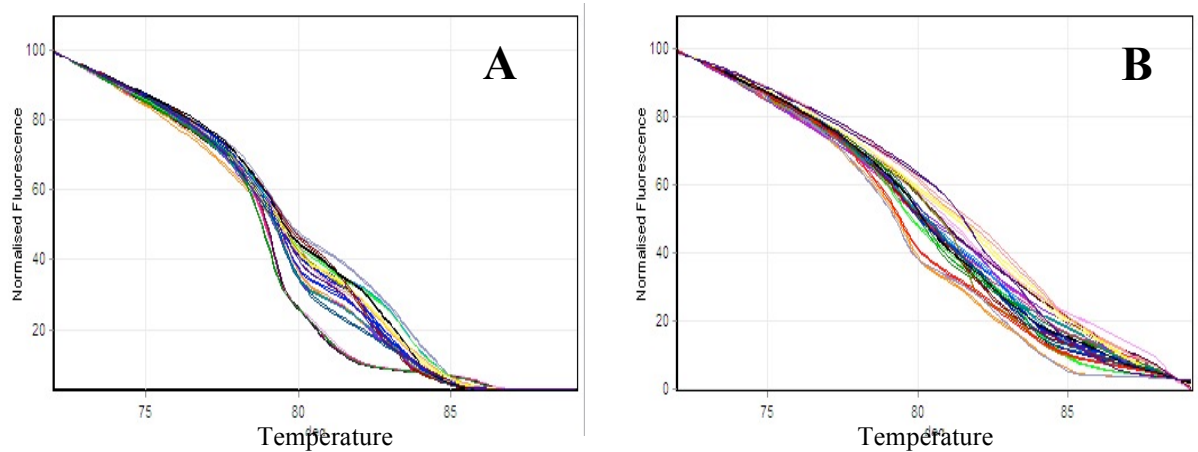
**Figure 3.7** Amplification profile of real-time PCR for two different groups (A) DNA extracted from pure culture (B) DNA extracted from stool samples.

The raw data obtained by HRM analysis for two groups of *C. difficile* using DNA extracted by two different methods are shown in Figure 3.8. Figure 3.8B shows that there is a greater range of fluorescence values and ending melt temperatures. This would be expected to affect the analysis downstream.



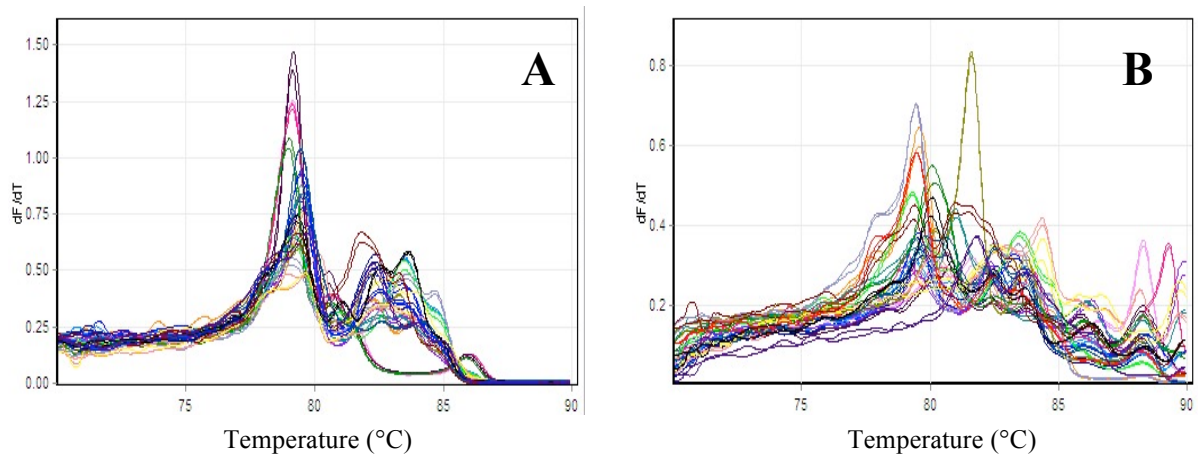
**Figure 3.8** HRM raw data showing different starting and ending points for 20 *C. difficile* clinical isolates using (A) DNA extracted from pure culture (B) DNA extracted from stool samples.

After normalization, Figure 3.9B shows that there has been a change to the melt-curve characterization of stool DNA compared to that of isolate DNA.



**Figure 3.9** HRM normalized graph for 20 *C. difficile* clinical isolates using (A) DNA extracted from pure culture and (B) DNA extracted from stool samples.

HRM melt peak analysis for 20 *C. difficile* clinical isolates using DNA extracted from pure culture and the same 20 isolates using DNA extracted directly from stool samples are shown in Figure 3.10.



**Figure 3.10** HRM melt peak analysis for 20 *C. difficile* clinical isolates using (A) DNA extracted from pure culture and (B) DNA extracted from stool samples.

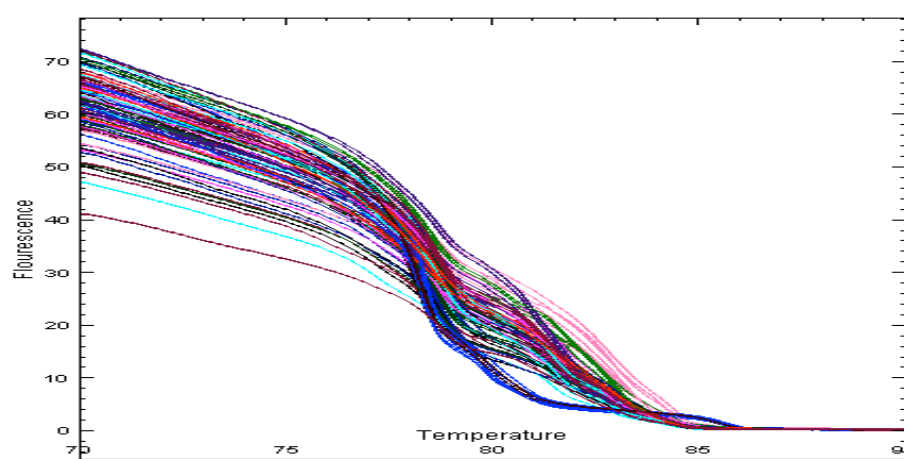
The HRM melt peaks obtained by using DNA extracted directly from stool samples (Figure 3.10A) have different peaks compared to the HRM melt peak obtained by using DNA extracted from pure culture of the same 20 *C. difficile* clinical isolates (Figure 3.10B). The differential height of peaks is almost half in Figure 3.10B compared to Figure 3.10A. This



probably indicates weak banding products so no further evaluation of stool DNA for HRM ribotyping was performed.

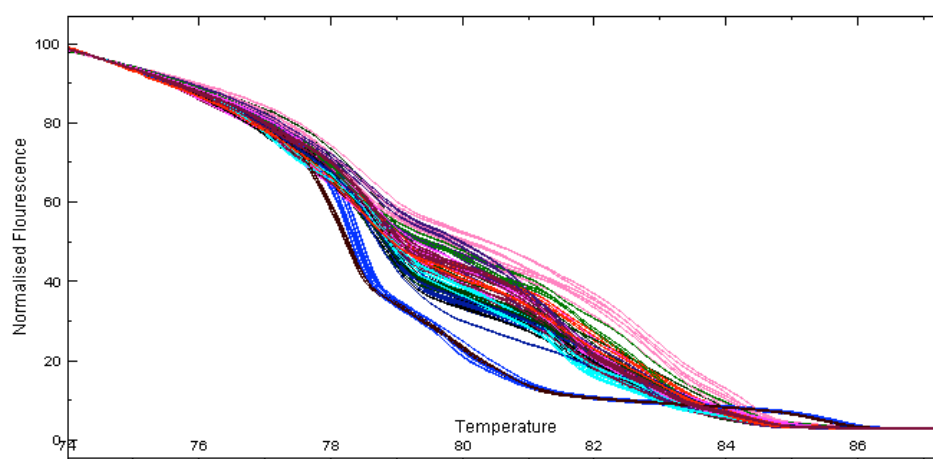
### 3.3.4 *ScreenClust* data analysis

*ScreenClust* HRM software was used to analyse whether clusters of isolates could be identified from the HRM data obtained by Rotor-Gene operating software. As previously stated ten clinical isolates (21, 23, 43, 45, 73, 79, 82, 83, 89 and 94) were excluded from analysis as they showed abnormal melt curves and high Ct value greater than 25. The HRM raw data for 83 *C. difficile* clinical isolates and five control strains are shown in Figure 3.11.



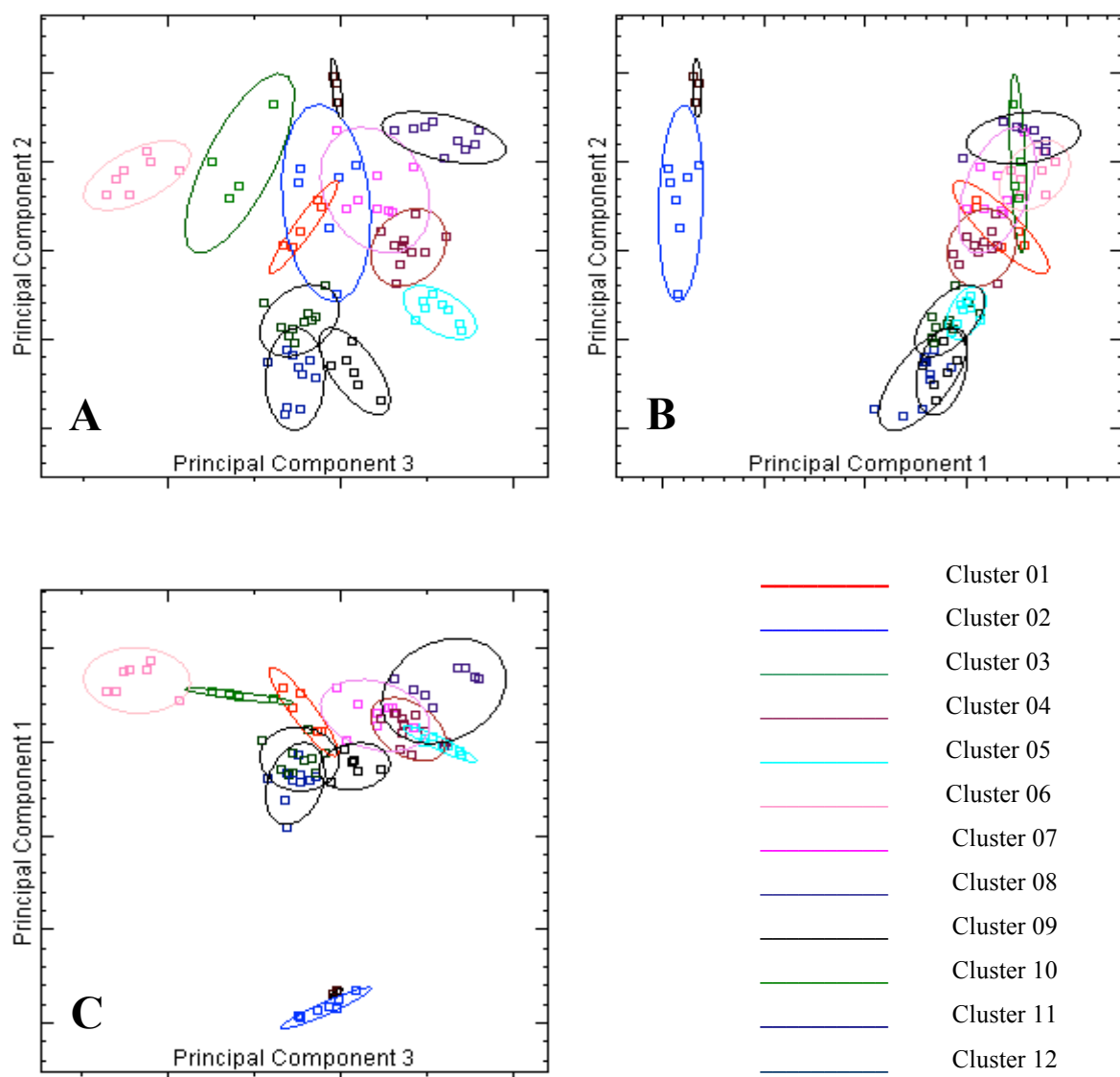
**Figure 3.11** HRM raw data showing different starting points of fluorescence for 83 *C. difficile* clinical isolates and 5 reference strains.

Figure 3.12 shows the normalisation curves of imported data from the HRM of 83 *C. difficile* clinical isolates and five control strains used in this study. It can be seen that the *Screenclust* software produces similar normalised melt curves in Figure 3.12 to that produced by Rotor-Gene software (Figure 3.4A).



**Figure 3.12** Normalized HRM curves for 83 *C. difficile* clinical isolate and five reference strains obtained.













After normalization using *ScreenClust* software a residual plot was generated by *ScreenClust*, the software defaults to use the data as a basis to produce principal component analysis by extracting a set of features for each curve. Principal component analysis selects the linear combination of the data vector that shows the most variation among the isolates as the first principal component (PC). The data is first analysed using unsupervised mode for clustering of the data. The software was able to associate isolates to 12 clusters and these clusters were each assigned a colour. These colours were used subsequently to label lines in the preceding Figures 3.3-3.14. The clusters of isolates are shown in Figure 3.13.



**Figure 3.13** Twelve clusters generated from principal component data analysis of 88 *C. difficile* isolates differentiated by colours created by software default according to genotype with 3 dimensional shapes A, B and C.

The isolates corresponding to each cluster are shown in Table 3.2.

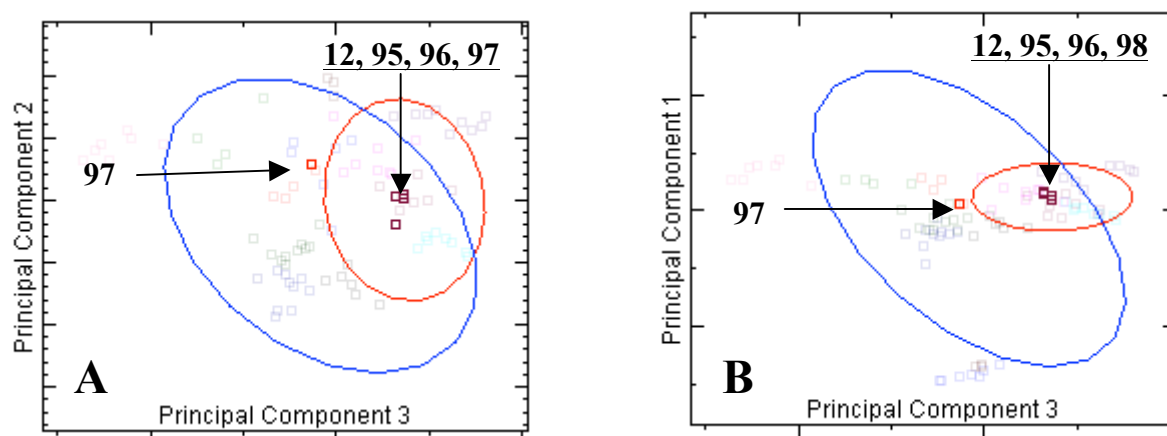
**Table 3.2** Eighty-three *C. difficile* isolate and five control strains differentiated into 12 clusters

Cluster No.	Colour	Representative Isolates
01		15, 90, 97*, 53, 60
02		04, 05, 08, 25, 46, 65, 70
03		16, 41, 69, 87
04		12, 20, 28, 33, 37, 76, 78, 80, 95*, 96*, 98*
05		14, 34, 40, 44, 71, 77, 84, 92
06		02, 17, 29, 32, 36, 42, 52
07		06, 30, 50, 54, 56, 61, 68, 91
08		03, 11, 18, 19, 22, 26, 31, 63, 81, 86
09		01, 07, 09, 27, 62, 74
10		10, 13, 24, 35, 49, 51, 64, 66, 72, 88
11		39, 48, 55, 57, 58, 59, 67, 75, 85
12		38, 47, 93

\* Ribotype 027 reference strains.

*ScreenClust* software grouped eight isolates into the same cluster (Table 3.2, cluster 4) with three ribotype 027 reference strains. As previously shown in Table 3.1, the homology of isolate 12 when examined by difference genotyping was 98% to the control strain R20291. The homology of these clustering isolates 20, 28, 33, 37, 76, 78 and 80 was found to be 63.6%, 46.6%, 59.8%, 90.5%, 58.5%, 52.7% and 48.4% respectively. Non of these other isolates were found in the same group when analysed by GelCompar II and in addition only isolates 12 and 78 showed the same toxin profile compared to ribotype 027 control strains (Figure 3.3). Isolate 12 had been shown to be ribotype 027, isolate 78 as stated above only had 52.7% homology with the control strain R20291. M7404 ribotype 027 control strain was not found in the same cluster with the other three 027 reference strains and it also has a different ribotype pattern as shown in Figure 3.3. The analysis was repeated in supervised

mode using R20291 ribotype 027 strain as a control. A subset of the clusters resulting from this analysis and containing 027 strains is presented in Figure 3.14.



**Figure 3.14** Two clusters generated from principal component data analysis using R20291 as a control strain in supervised mode and the clinical isolate 12 and the four ribotype 027 strains.

In Figure 3.14, isolate 12 shows close association with the control strains.

### 3.3.5 Measurement of discriminatory power

Using Simpson's index of diversity to measure the discriminatory power of PCR-ribotyping and HRM using *ScreenClust* software for data analysis with the same total number of isolates (88 isolates) showed PCR-ribotyping to have an index of 0.902 and HRM had an index of 0.928.

### 3.4 Discussion

Typing of *C. difficile* using the variation in ISR was first described by Gurtler (1993). The current study uses real-time PCR to produce the ISR amplicons and is the first time that HRM with *ScreenClust* software has been used to analyse ISR's for ribotyping. It has been reported that bands generated by PCR-ribotyping in *C. difficile* may only differ in size by less than 10 nucleotides (Gurtler, 1993). Thus one limitation of PCR-ribotyping is that agarose gel separation of PCR products may lack resolution. The bands generated by early PCR-ribotyping methods exhibited bands with high and close molecular masses, thus some patterns were difficult to read on the agarose gel electrophoresis (Gurtler, 1993, Kostman et al., 1992). A modification of the method was developed in order to reduce the size of PCR generated fragments by choosing new primers closer to the ISR (O'Neill et al., 1996). In this study we used PCR-ribotyping primers that were located closer to the ISR and give shorter PCR products (Bidet et al., 1999). This method gave clear separate bands ranging from 260 to 2176 bp when separated on agarose gel electrophoresis. The modified PCR-ribotyping method of Bidet et al. (1999) showed high discriminatory power calculated as 0.902 by Simpson's index. PCR-ribotyping has been considered to be a more discriminatory approach than arbitrary-primer PCR (AP-PCR) for typing *C. difficile* (Collier et al., 1996) but less than PFGE in discriminatory power (Brazier, 1998).

GelCompar II was used to analyse 88 *C. difficile* isolates and four ribotype 027 strains resulting in the identification of a clinical isolate (isolate number 12) with an identical ribotyping pattern to three control strains and HRM genotyping confidence of 98%. This isolate has a toxin gene profile identical to those of the control 027 group. The similarity of this isolate to control isolates was confirmed by *ScreenClust* where this isolate was found to cluster with the 027 group. Warny et al. (2005) showed that M7404 (Canadian ribotype 027 strain) has a slightly different PFGE pattern, but did not mention if the ribotype pattern

varied. In this study we found that M7404 had a slightly different ribotyping pattern but still grouped with 027 strains using GelCompar II. Similarity using HRM ribotyping, M7404 did not cluster with the control 027 strains. It would be interesting to investigate further isolates from Canada to see if they consistently are identified within a unique cluster.

In the study performed by Stabler et al. (2006) comparative phylogenomics was applied to model the phylogeny of *C. difficile* including 75 diverse isolates. Four identified distinct statistically supported clusters were identified and among these only 19.7% of characteristics were shared by all strains confirming that this enteric species readily undergoes genetic exchanges. Further to this, Stabler et al. (2010) stated that “*C. difficile* has a highly fluid genome with multiple mechanisms to modify its genetic content and is continuing to evolve in our hospitals influenced by environmental changes and human activity”.

Comparing the historical CD196 strain with the modern hypervirulent strain R20291 showed that this modern strain has five additional genetic regions compared to its historic counterpart. Furthermore both 027 strains have an additional 234 genes compared to *C. difficile* 630 strain, which may account for the marked increase in disease capability (Stabler et al., 2009). However, in bacteria there are other mechanisms of genetic variation, and perhaps counter intuitively gene re-arrangements and gene loss can be equally important in the evolution of virulence (Pallen and Wren 2007).

Isolate number 90 also grouped with the 027 controls when analysed by GelCompar II, however it has a different toxin gene profile. This isolate did not cluster with 027 controls when analysed by *ScreenChust* software. This reinforces the importance of firstly

determining what toxin genes are present. Isolates with suspicious toxin gene profiles may then be further examined by ribotyping methods. Figure 3.3 lists the results of CTA testing of isolates. If we look at particular groups, for instance the first two isolates in group four, we can see that although there is greater than 90% homology for these two isolates (75 and 85), they gave different CTA results. CTA testing has been found to be poorly sensitive at 76.7% (Peterson et al., 2007) and 67.2% (Stamper et al., 2009a) and this would seem to be confirmed by the results of the current study.

HRM analysis allows for both visual- and auto-calling of genotypes. Results can be viewed as either a normalized melt plot or a difference plot. Normalized curves of the 88 *C. difficile* including four ribotype 027 strains shown in Figure 3.4A demonstrated variation of curves between isolates. In Figure 3.4B it can be noted that that isolate number 12 has a melt curve similar to that of ribotype 027 strains (CD196, KI AND R20291), however the Canadian strain M7404 has a slightly different melt curve as shown in Figure 3.4B. This finding was confirmed by difference plots when using the UK strain (R20291) as control for genotyping as shown in Figure 3.5B and Table 3.1. The difference plots provide an alternative view of the differences between melt curve transitions. It should be noted that it would not be expected to get the same grouping of isolates using HRM analysis of PCR-ribotyping and gel separation of PCR-ribotyping products. The HRM curve is produced as a combination of all PCR fragmented generated rather than separate analysis of each fragment. It would be interesting to see sequencing of the M7404 strain in order to compare the inter spacer region of this strain to other 027 strains. From the HRM data it would seem there are a number of differences (Figure 3.5B).

Twelve clusters were generated from principle component analysis of HRM data when analysed in unsupervised mode. Isolate number 12 was in the same cluster as the three



ribotype 027 reference strains. Even when supervised mode is used in *ScreenClust* M7404 does not cluster with the other 027 strains. This raises the possibility that *ScreenClust* might be useful to investigate isolates from Canada and then M7404 would be selected as control in supervised mode. It was found that an isolate with a similar toxin profile (isolate 78) also clustered with the control 027 strains. It would be important to consider the *tcdC* deletion carry as important additional information. Isolate 78 was found to have a deletion in *tcdC* (Chapter 2, Figure 2.5), however it was not the signature deletion of other 027 strains.

Once DNA has been extracted, PCR-ribotyping requires around eight hours to achieve a result, while HRM using *ScreenClust* for data analysis can be performed in less than two hours. Although it would be desirable to be able to perform HRM ribotyping directly from stool, this was not expected to be feasible. As the result showed, the Ct values were lower from stool and melt peaks less defined. Additionally if HRM ribotyping is performed directly from stool, it could be expected that the ISR of other closely related *Clostridium spp.* may also be amplified thus further confusing the melt peaks obtained.

Using Simpson's index of diversity to measure and compare the discriminatory power between PCR-ribotyping and HRM analysis developed in this study showed PCR-ribotyping to have an index of 0.902 and HRM of 0.928. This index indicates that if two strains were sampled randomly from the population by PCR ribotyping, then on 90% of occasions they would fall into different types. HRM analysed through *ScreenClust* has a similar discriminatory value (92%) to that of PCR ribotyping. Further work would be required in a clinical setting to see if the HRM ribotype clusters are clinically relevant.

In conclusion, *ScreenClust* software was capable of detecting, clustering and calling genotypes from various HRM data sets in a short time. The difference in the Ct values and

curve characteristic of the real-time PCR obtained from isolate DNA would suggest that HRM PCR-ribotyping cannot be performed directly from stool. However since PCR toxin typing is suggested to be performed first and can be performed the same day as stool receipt, it is feasible to perform culture on stool from suspicious PCR toxin types and then batch test these isolates in HRM ribotyping.

## **CHAPTER 4**

### **General discussion and future directions**

#### 4. General discussion and future directions

The emergence and spread of the epidemic strain PCR ribotype 027 has caused many outbreaks around the world (McDonald et al., 2005, Kuijper et al., 2006, Kuijper et al., 2008). Outbreaks are continuing to occur in Canada (CBC, 2011), and Australia (Wang et al., 2011). Muto et al. (2007) have published methods used to control outbreaks in the USA and they stated that early identification of cases was important for effective control. The limitations of traditional testing for CDAD are due to the lack of sensitivity when detecting toxin in stool, the slowness of testing when performing cytotoxic culture and the lack of specificity when performing *C. difficile* isolation. Laboratory testing for *C. difficile* is being facilitated by the next generation of tests based on PCR methodologies.

Currently in the USA many of the commercially available Federal Drug Administration (FDA) approved PCR tests are based on detecting the TcdB gene. An example of the danger of using a PCR test based on one gene was seen when *Neisseria gonorrhoea* mutant isolates were no longer detected by commercial assays (Farrell, 1999, Tabrizi et al., 2004). It is not only commercial assays that are prone to this problem as was described recently when an in-house assay for *N. gonorrhoea* also failed to detect an isolate due to sequence variation of the target gene (Whiley et al., 2004). In the current study, significant sequence variation was found when the primers published by Wroblewski et al. (2009) were compared to recently published sequences of *C. difficile*. As the assay used in the current study was developed in-house, the primers were able to be quickly redesigned and applied.

To overcome the problems of testing for one gene, the current study chose a multiplex PCR. The current study showed that the multiplex real time PCR for detection of *C. difficile* toxin genes *tcdA* and *tcdB* as well as binary toxin *cdtA* and *cdtB* using DNA extracted by an automated system gave reliable results when compared to CTA and

conventional PCR. Including PCR primers for binary toxin genes in *C. difficile* multiplex testing would seem to be important especially as *tcdA*- *tcdB*- binary toxin positive isolates have been reported and that binary toxin is a suspected virulence factor in *C. difficile* (Barbut et al., 2005).

TcdA gene negative, TcdB gene positive isolates have been reported (Lyerly et al., 1992). On further examination of these isolates it was found that parts of *tcdA* were truncated. The Wroblewski et al. PCR primers for *tcdA* used in the current study detect *tcdA* regardless of whether there is a truncation present. It was interesting to review the results of PCR conventional testing where some bands were weak. Thus the rationale for testing using both *tcdA* and *tcdB* primers was decided as a way of controlling for both. It could be seen in the current study that signal strength also varied in real-time PCR. Thus testing for both *tcdA* and *tcdB* could be seen as creating a control for each gene in case one gene produced weak signals.

Real-time PCR is able to produce results in around a quarter of the time for conventional PCR. A meta-analysis has been performed to determine the diagnostic accuracy of real-time PCR (Deshpande et al., 2011). This meta-analysis reported that real-time PCR has a high sensitivity and specificity when compared with cell culture cytotoxicity neutralisation assay (CCCNA) or anaerobic toxigenic culture (TC) (Deshpande et al., 2011). Interestingly, none of the in-house real time PCR reported in the meta-analysis are approved by the FDA. This is probably because extensive testing is required before FDA approval is granted and this is beyond the financial ability of most laboratories. A study reported by Stamper et al. (2009a) reported that upon initial testing, the BD GeneOhm Cdiff assay (FDA) compared favourably to the Wampole *C. difficile* Toxin B test with a sensitivity and specificity of 90.9% and 95.2%, respectively. When both assays were compared to anaerobic toxigenic

culture, the sensitivity of the BD GeneOhm Cdiff assay dropped to 83.6%, but its specificity improved to 98.2% (Stamper et al., 2009a). More recently a study performed by Knetsch et al. (2011) compared the diagnostic values of three in-house real-time PCRs and a commercially available BD GeneOhm Cdiff assay, using the cytotoxigenic culture as a gold standard. They showed that the sensitivity of the in-house real-time PCRs was better than the BD GeneOhm test, but in contrast the specificity of the commercial test was superior to the in-house test (Knetsch et al., 2011). The sensitivity and specificity of the in-house PCR assay of the current study should be compared to commercial assays as this will determine if the in-house assay produces superior results. It is important that laboratories be encouraged to benchmark their assays so that the best assay for any particular patient population be used and so that commercial manufacturers are encourage to improve their assays if they are found to underperform in-house assays.

Future studies should be performed to work out the true sensitivity and specificity of the real-time testing platform described in this study. To do this would require that the best current gold-standard be used. Unfortunately the current best gold-standard is cytotoxigenic culture but this takes at least four days. Stool samples would be tested prospectively rather than the retrospective collection of the current study. Future studies should be performed in conjunction with physicians so that good clinical data can be collected. Clinical information will help to identify those patients with severe CDI and ensure that laboratory testing correlates with clinical finding.

Ribotype 027 strains have been associated with severe CDI. It is important to quickly identify these strains before they cause hospital outbreaks. The publication of de Boer et al. (2010) reported the first Taqman based real-time PCR capable of detecting the *tcdC*  $\Delta$ 117 mutation in ribotype 027 strains without the need of melting curve analysis. We have

shown the utility of this method in our study and have additionally added the scatter plotting analysis. Knetsch et al. (2011) reported a sensitivity and specificity of 98.0% and 90.7% respectively for a real-time PCR targeting the *tcdC* frameshift mutation at position 117 ( $\Delta$ 117 PCR) in stool samples when compared with PCR-ribotyped CYTGC-positive samples. Future work by our group would aim to determine the sensitivity and specificity of the de Boer et al. method in comparison to the Knetsch et al. method. The real-time PCR assay targeting  $\Delta$ 117 using DNA extracted by an automated system in our study would seem to give reliable results when using scatter plotting software for clustering 027 strains in this study.

In the second part of the current study, *ScreenClust* software was demonstrated to be capable of detecting, clustering and calling genotypes from various HRM data sets in a short time. The difference in the Ct values and curve characteristic of the real-time PCR obtained from isolate DNA and stool DNA would suggest that HRM PCR-ribotyping cannot be performed directly from stool. However since PCR toxin typing is suggested to be performed first and can be performed the same day as stool receipt, it is feasible to perform culture on stool from suspicious PCR toxin types and then batch test these isolates in HRM ribotyping.

The current study also provides evidence that there are other isolates present in the study population with a toxin profile similar to that of ribotype 027/NAP1, despite being of different ribotype. These other isolates could be important sources of further outbreaks and should be followed in the future to watch for changes in clusters of isolates that may be caused by strains with binary toxin genes. He et al. (2010) stated that “*Clostridium difficile* has rapidly emerged as the leading cause of antibiotic-associated diarrheal disease, with the transcontinental spread of various PCR ribotypes, including 001, 017, 027 and 078.

However, the genetic basis for the emergence of *C. difficile* as a human pathogen is unclear”.

In Europe other strains have emerged to become important nosocomial pathogens. In a study performed in 2002 more than 66 different PCR ribotypes were reported from 354 toxinogenic *C. difficile* isolates. “The distribution was varied markedly among hospitals and countries. Of all toxinogenic isolates, ribotype 001 was the most common (13%), followed by ribotype 014 (9%); ribotypes 002, 012, 017, 020, and 027 were each found in 6% of toxinogenic isolates, whereas ribotype 078 was found in 3% of toxinogenic isolates” (Freeman et al., 2010). Our study was unable to identify these other possible outbreak strains so future studies would obtain control isolates from these other epidemiologically important ribotypes and would further study possible de-novo identified clusters.

In conclusion, this study has adapted a real-time PCR for *C. difficile* toxin gene testing to direct testing of stool via automated DNA extraction. This PCR has produced reliable results and should be further investigated in a prospective study. Secondly we have found that scatter plotting software identified the mutation that is signature of the hypervirulent strain and may provide same day information crucial for rapid identification and infection control. Finally HRM analysis of PCR-ribotyping produces clusters of isolates that identified the epidemic strain in a patient. This novel typing technique shows the potential to provide same day typing for bacterial isolates. This novel approach to typing should also be further investigated in a prospective study to show whether it aids in the control of *C. difficile* infection.



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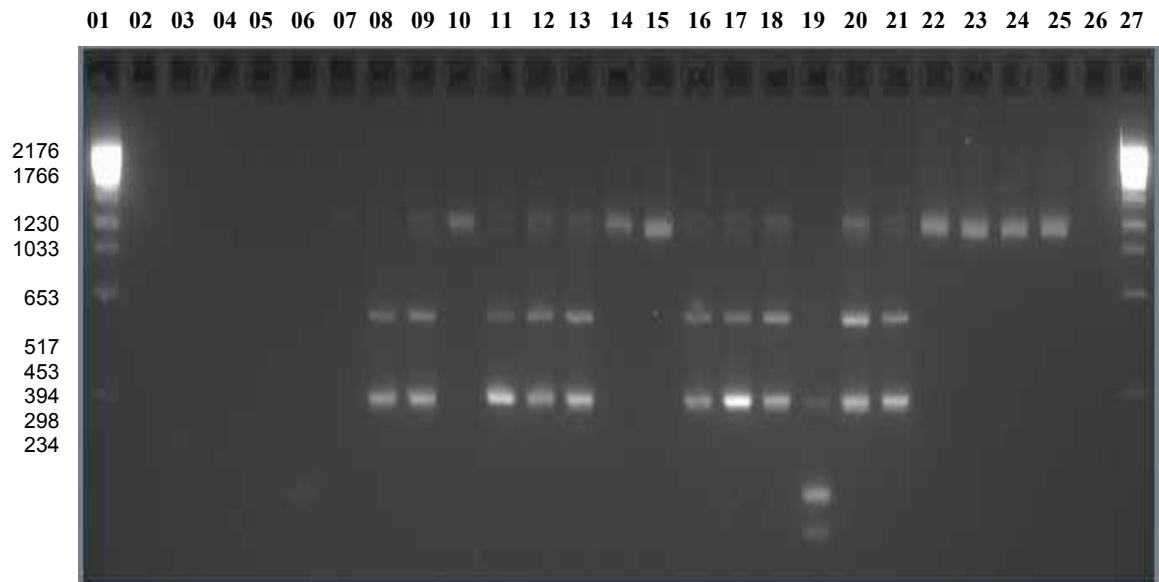
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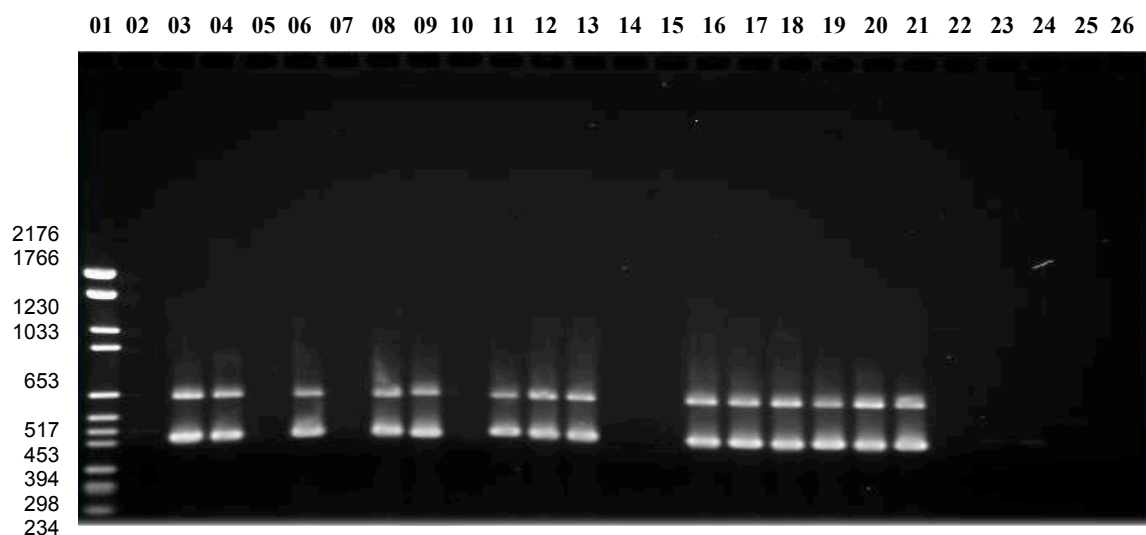
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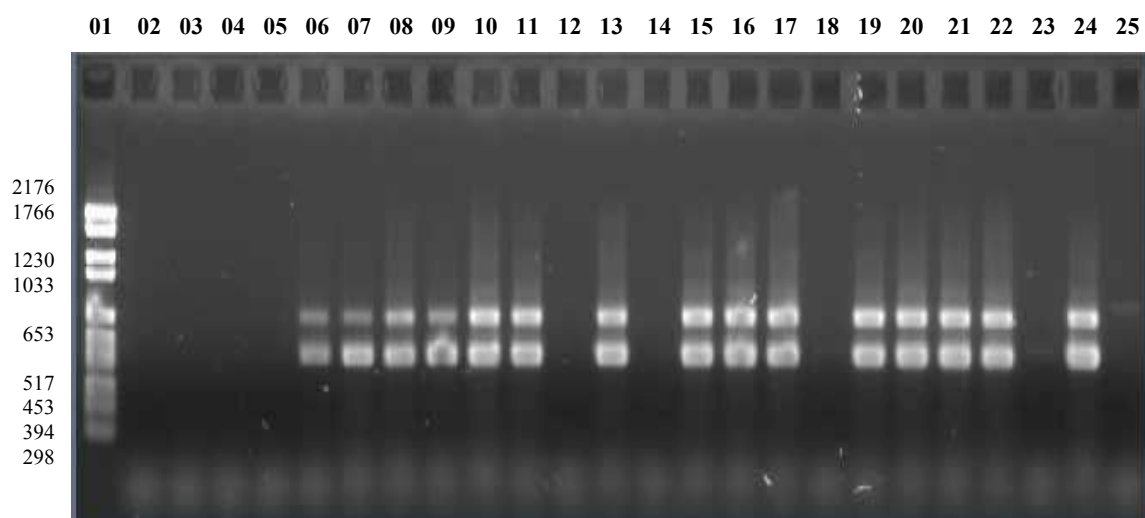
**Appendix A:** Gel images for Conventional PCR using DNA extracted from pure culture.



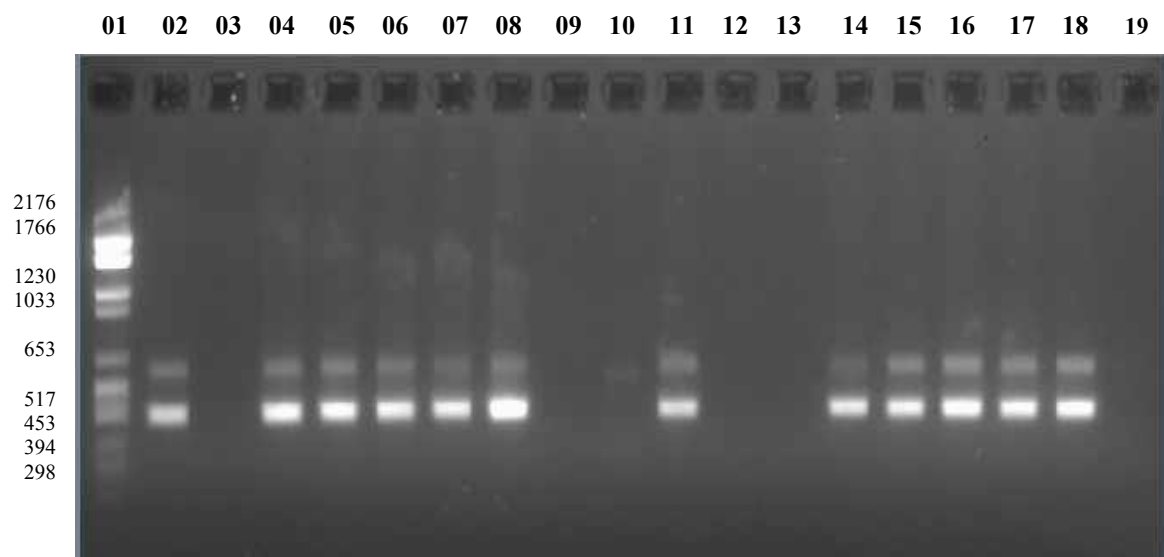
**Figure A.1** Amplification of 16s rDNA (1062 bp), *tcdA* (629 bp), *tcdB* (410 bp), *cdtA* (221 bp) and *cdtB* (262 bp) by multiplex conventional PCR in 13 *C. difficile* isolates. Lane 1 and 27 are DNA marker VI (154-2176 bp); lanes 2-25 representing *C. difficile* isolates number 25-48; lane 26 is NTC.



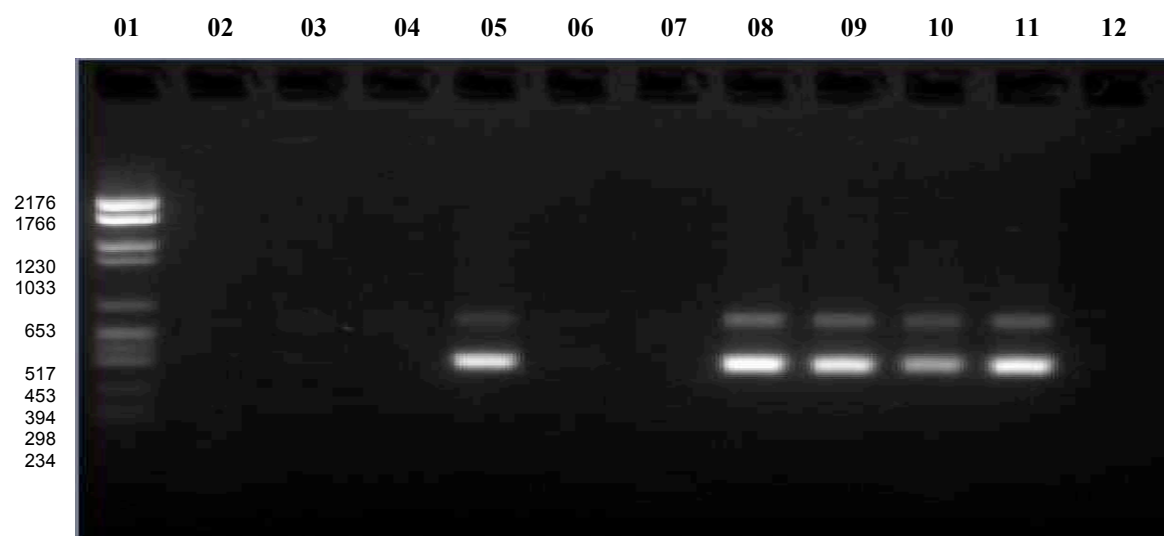
**Figure A.2** Amplification of *tcdA* (629 bp) and *tcdB* (410 bp) by 2-plex conventional PCR in 24 *C. difficile* isolates. Lane 1 is DNA marker VI (154-2176 bp); lanes 2-25 representing *C. difficile* isolates number 25-48; lane 26 is a NTC.



**Figure A.3** Amplification of *tcdA* (629 bp) and *tcdB* (410 bp) by 2-plex conventional PCR in 23 *C. difficile* isolates. Lane 1 is DNA marker VI (154-2176 bp); lanes 2-24 representing *C. difficile* isolates number 49-71; lane 25 is a NTC.

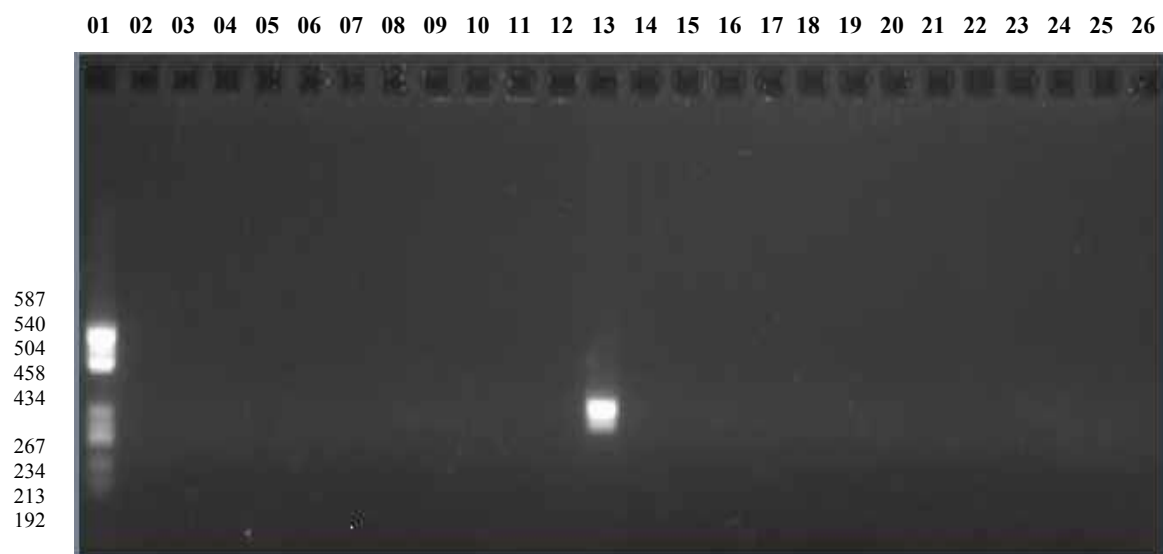


**Figure A.4** Amplification of *tcdA* (629 bp) and *tcdB* (410 bp) by 2-plex conventional PCR in 17 *C. difficile* isolates. Lane 1 is DNA marker VI (154-2176 bp); lanes 2-18 representing *C. difficile* isolates number 72-88; lane 19 is NTC.

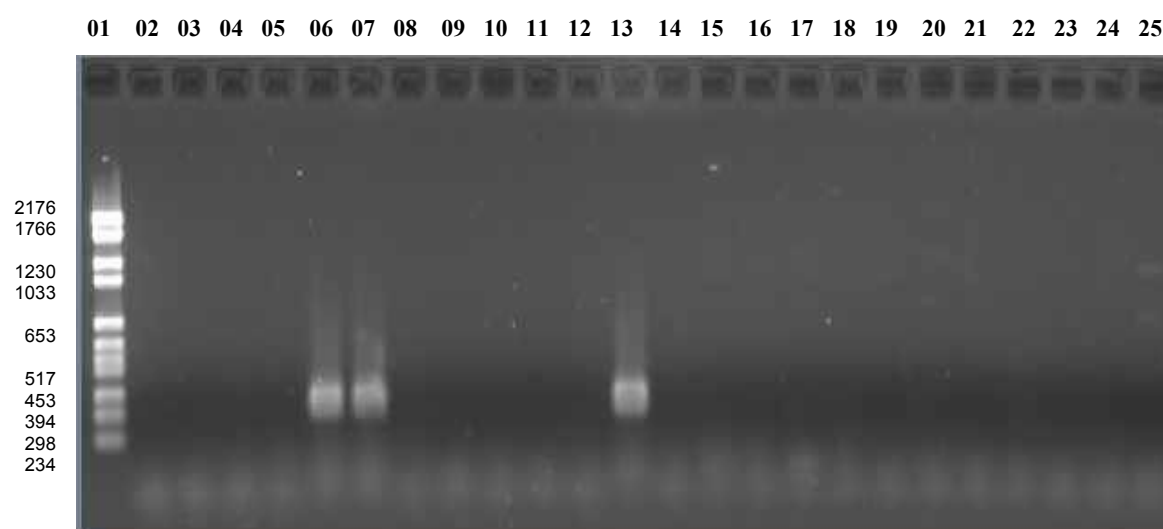


**Figure A.5** Amplification of *tcdA* (629 bp) and *tcdB* (410 bp) by 2-plex conventional PCR. Lane 1 is DNA marker VI (154-2176 bp); lanes 02-07 representing *C. difficile* isolates number 89-94; lanes 08-11 *C. difficile* ribotype 027 controls 95, 96, 97 and 98; (KI, R20291, M7404 and CD196) respectively; lane 19 is a NTC.

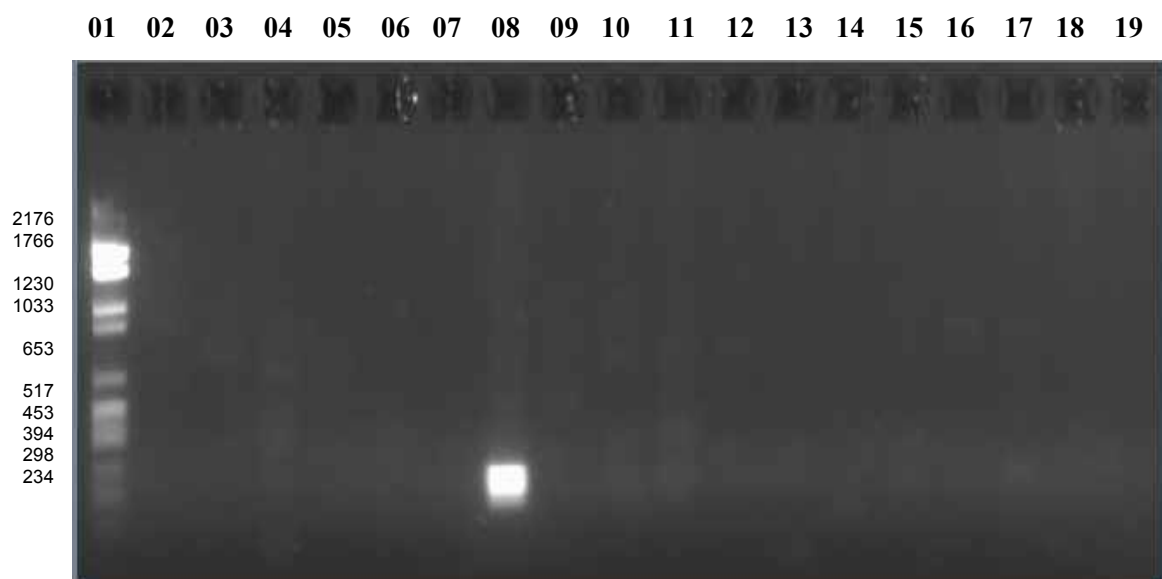




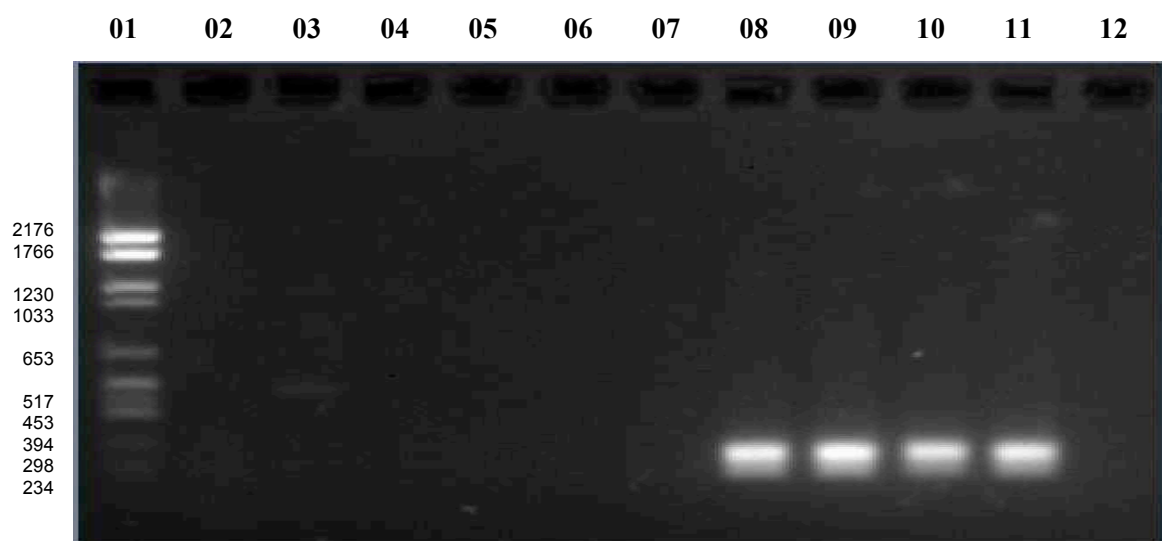
**Figure A.6** Amplification of *cdtA* (221 bp) and *cdtB* (262 bp) by 2-plex conventional PCR. Lanes 1 is DNA marker V (8-587 bp); lanes 2-25 representing *C. difficile* isolates number 01-24; lane 26 is a NTC.



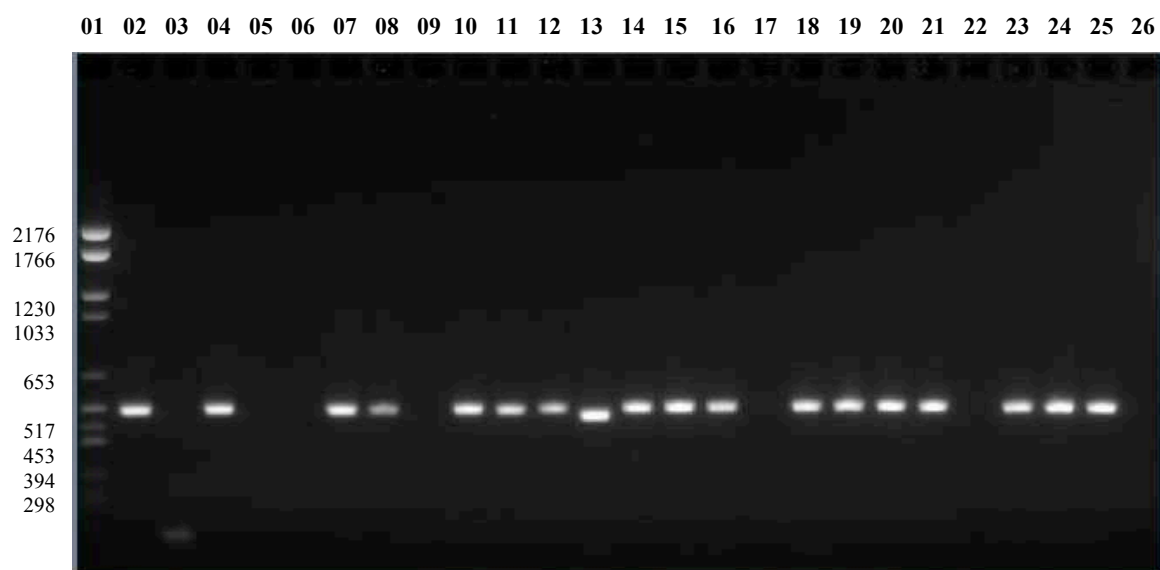
**Figure A.7** Amplification of *cdtA* (221 bp) and *cdtB* (262 bp) by 2-plex conventional PCR. Lanes 1 is DNA marker VI (154-2176 bp); lanes 2-24 representing *C. difficile* isolates number 49-71; lane 25 is a NTC.



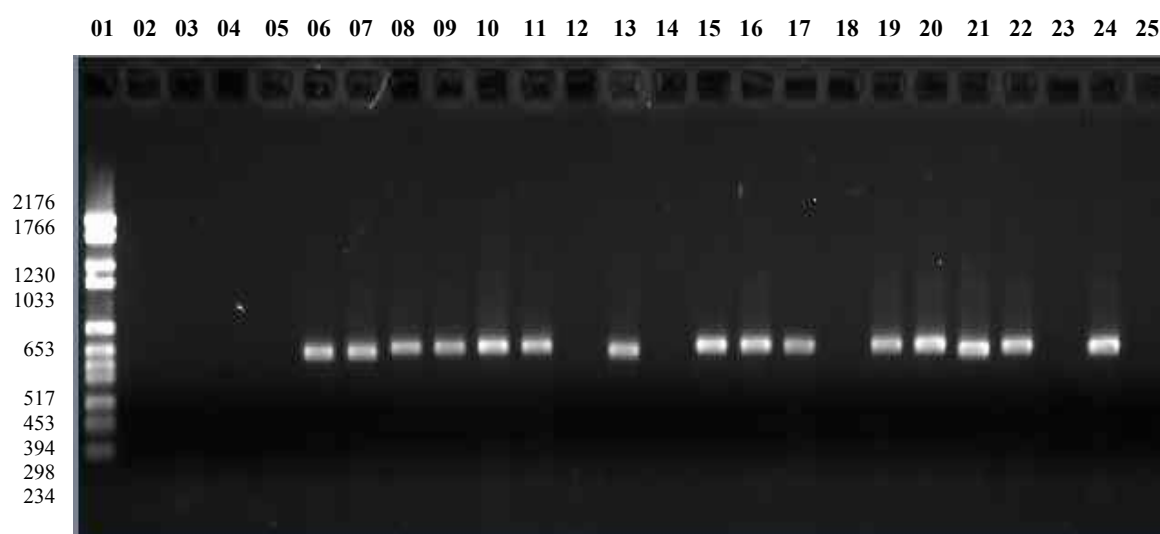
**Figure A.8** Amplification of *cdtA* (221 bp) and *cdtB* (262 bp) by 2-plex conventional PCR. Lanes 1 is DNA marker VI (154-2176 bp); lanes 2-18 representing *C. difficile* isolates number 72-88; lane 19 is a NTC.



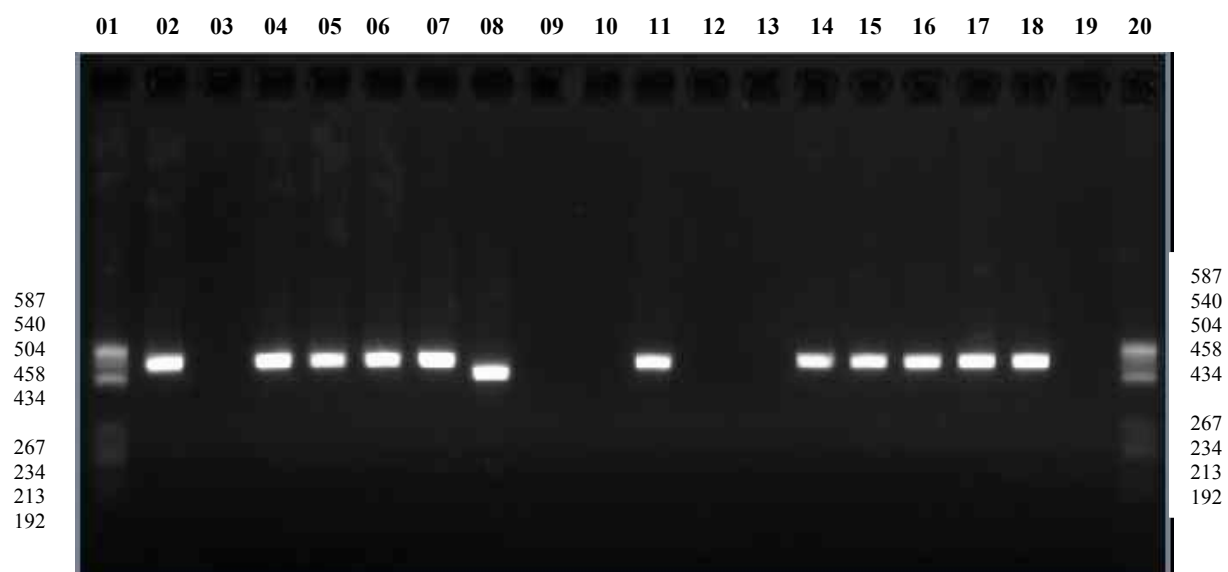
**Figure A.9** Amplification of *cdtA* (221 bp) and *cdtB* (262 bp) by 2-plex conventional PCR. Lane 1 DNA marker VI (154-2176 bp); lanes 02-07 representing *C. difficile* isolates number 89-94; lanes 08-11 *C. difficile* ribotype 027 controls 95, 96, 97 and 98; (KI, R20291, M7404 and CD196) respectively; lane 12 is a NTC.



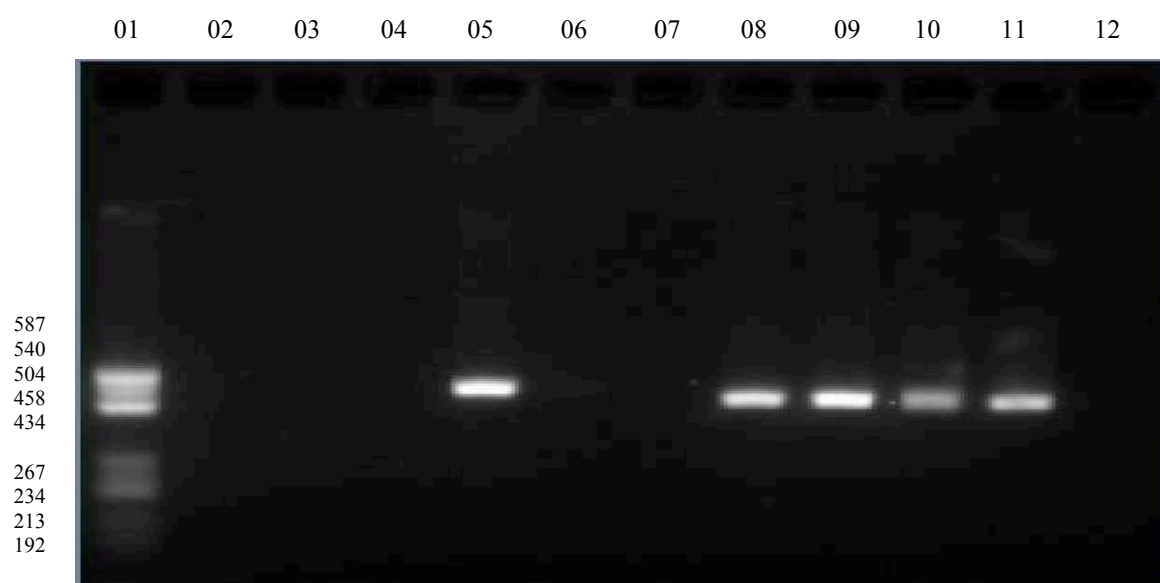
**Figure A.10** Amplification of *tcdC* (475 bp) by conventional PCR. Lanes 1 is DNA marker VI (154-2176 bp); lanes 2-25 representing *C. difficile* isolates number 1-24; lane 26 is a NTC.



**Figure A.11** Amplification of *tcdC* (475 bp) by conventional PCR. Lanes 1 is DNA marker VI (154-2176 bp); lanes 2-24 representing *C. difficile* isolates number 49-71; lane 25 is a NTC.

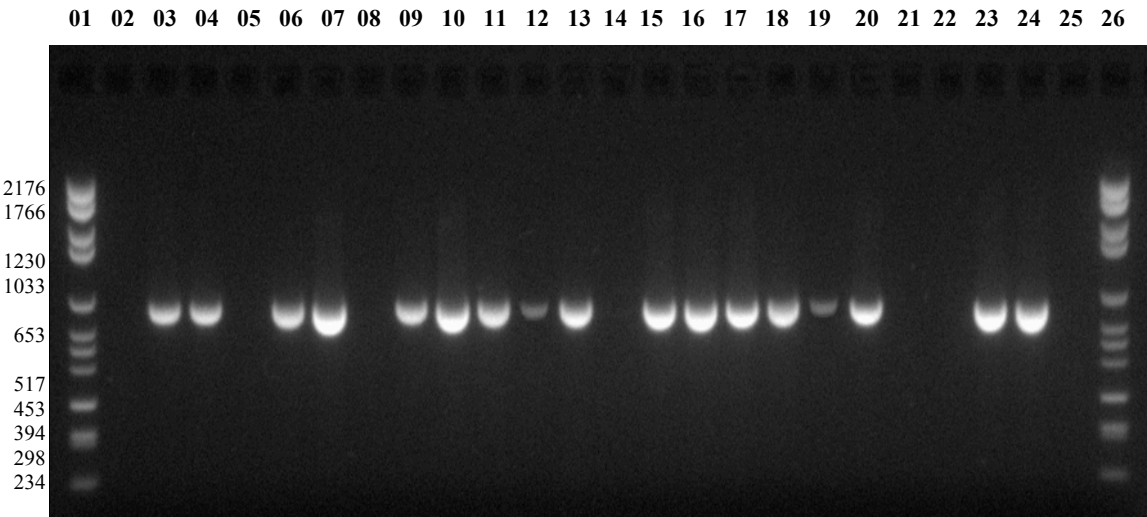


**Figure A.12** Amplification of *tcdC* (475 bp) by conventional PCR. Lanes 1 and 20 is DNA marker V (8-587 bp); lanes 2-18 representing *C. difficile* isolates number 72-88; lane 19 is a NTC.

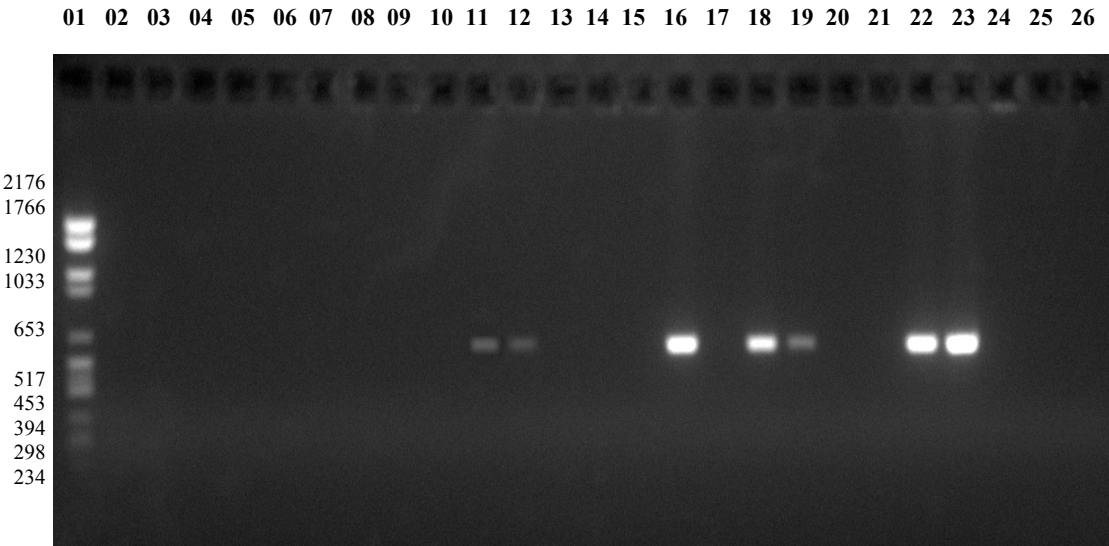


**Figure A.13** Amplification of *tcdC* (475 bp) by conventional PCR. Lane 1 DNA marker V (8-587 bp); lanes 02-07 representing *C. difficile* isolates number 89-94; lanes 08-11 *C. difficile* ribotype 027 controls 95, 96, 97 and 98; (KI, R20291, M7404 and CD196) respectively; lane 12 is a NTC.

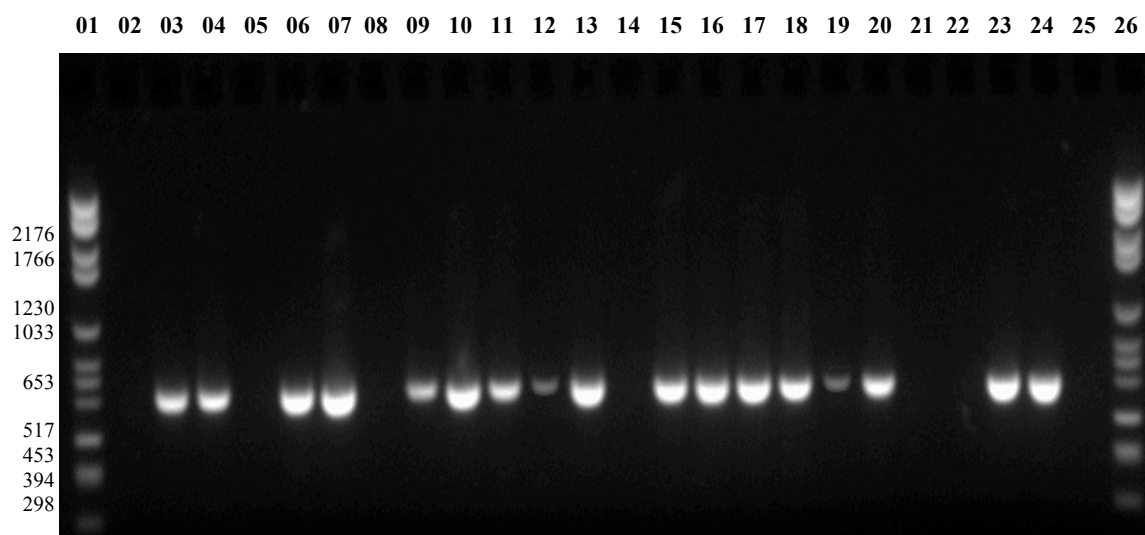
**Appendix B:** Gel images for cconventional PCR using DNA extracted from stool samples



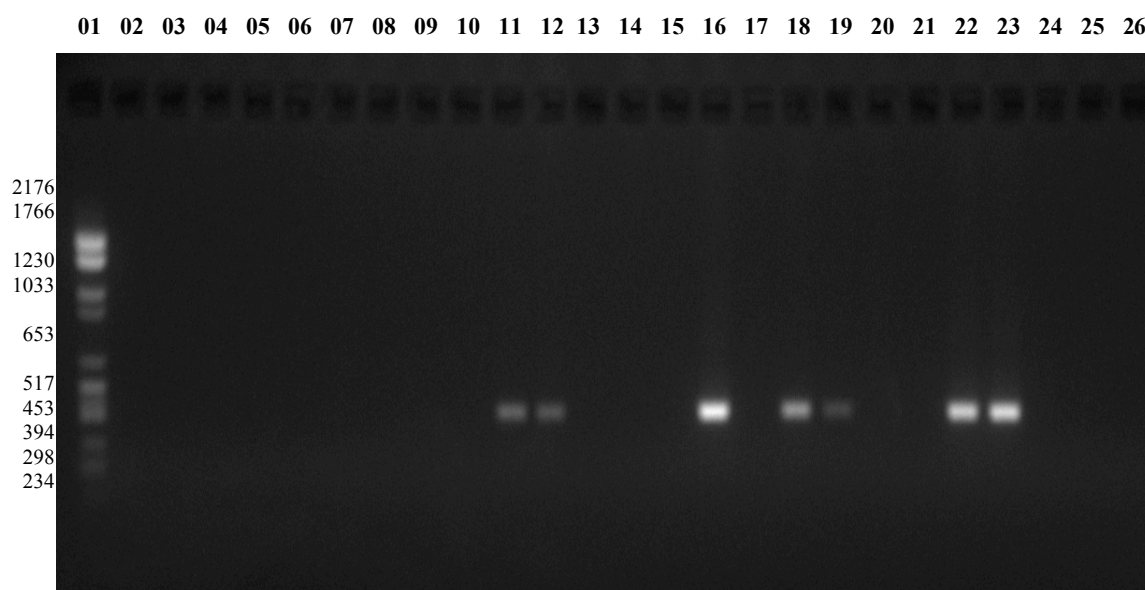
**Figure B.1** Amplification of *tcdA* resulting in a 629 bp product by conventional PCR using stool DNA. Lanes 1 and 26 are DNA molecular weight marker VI (154-2176 bp); Lanes 02-24 representing stool samples number (52, 53, 54, 55, 56, 57, 59, 63, 64, 66, 69, 71, 74, 75, 76, 77, 78, 79, 81, 82, 83, 84 and 86); lane 25 negative control.



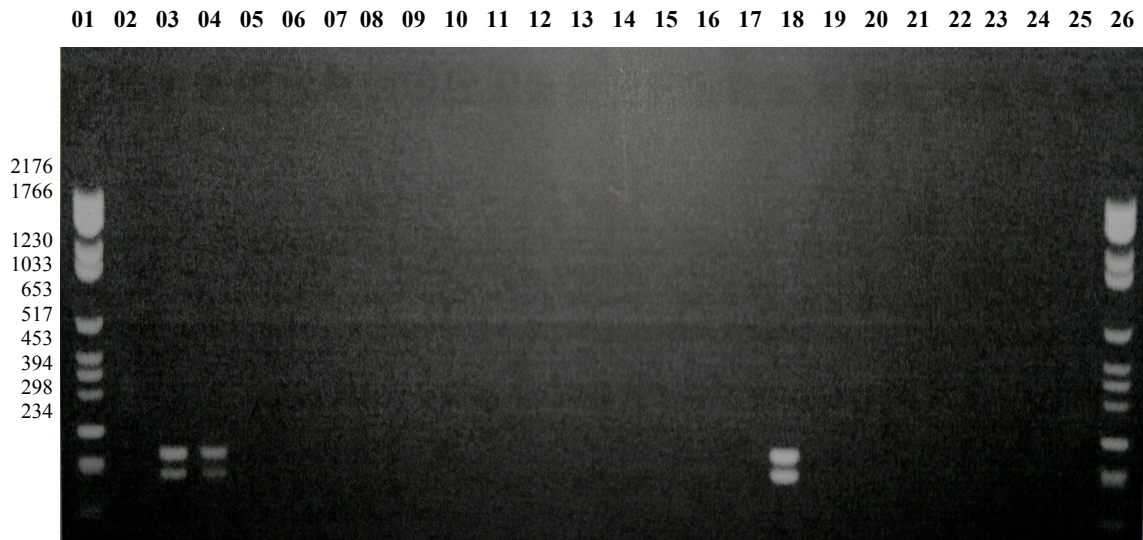
**Figure B.2** Amplification of *tcdA* resulting in a 629 bp product by conventional PCR using stool DNA. Lanes 1 is DNA molecular weight marker VI (154-2176 bp); Lanes 02-25 representing stool samples number (3, 6, 15, 16, 21, 24, 28, 30, 33, 34, 45, 47, 48, 61, 62, 65, 72, 73, 80, 85, 87, 88, 90 and 91); lane 26 is a negative control.



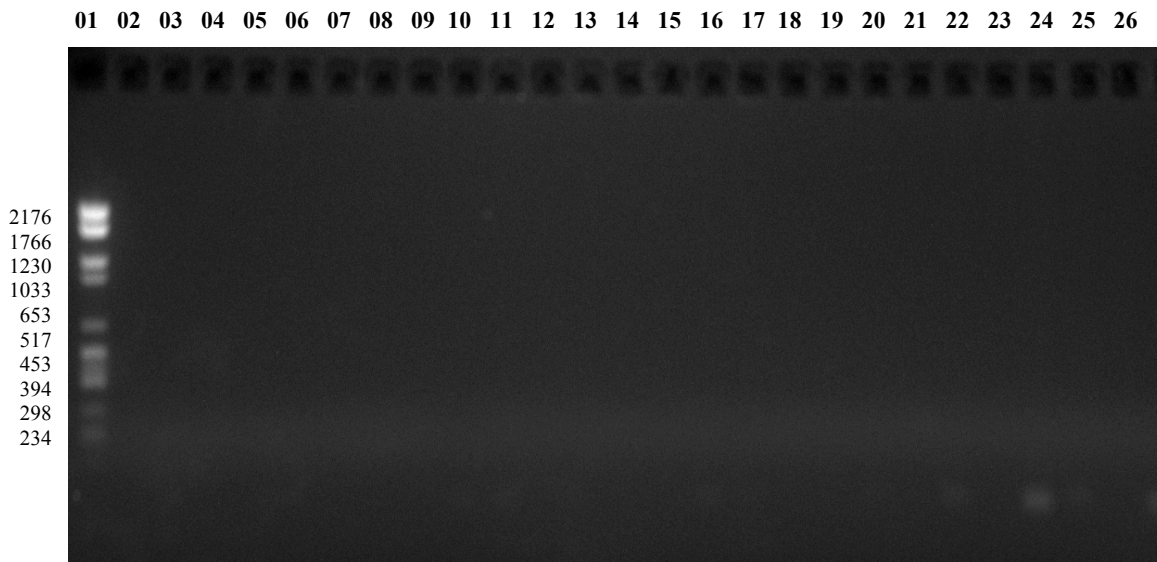
**Figure B.3** PCR products from amplification of *tcdB* (410 bp) by conventional PCR using stool DNA. Lanes 1 and 26 are DNA molecular weight marker VI (154-2176 bp); Lanes 02-24 representing stool samples number (52, 53, 54, 55, 56, 57, 59, 63, 64, 66, 69, 71, 74, 75, 76, 77, 78, 79, 81, 82, 83, 84 and 86); lane 25 negative control.



**Figure B.4** PCR products from amplification of *tcdB* (410 bp) by conventional PCR using stool DNA. Lanes 1 is DNA molecular weight marker VI (154-2176 bp); Lanes 02-25 representing stool samples number (3, 6, 15, 16, 21, 24, 28, 30, 33, 34, 45, 47, 48, 61, 62, 65, 72, 73, 80, 85, 87, 88, 90 and 91); lane 26 is a negative control.



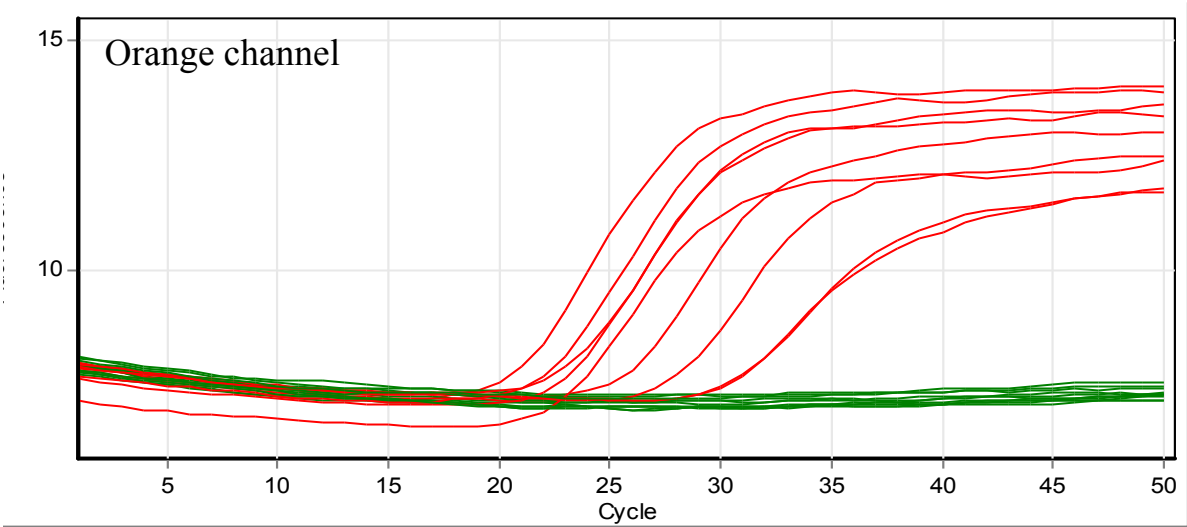
**Figure B.5** Amplification of *cdtA* 221 bp products and *cdtB* 262 bp products by conventional PCR using stool DNA in 23 stool samples. Lanes 1 and 26 are DNA molecular weight marker VI (154-2176 bp); Lanes 02-24 representing stool samples number (52, 53, 54, 55, 56, 57, 59, 63, 64, 66, 69, 71, 74, 75, 76, 77, 78, 79, 81, 82, 83, 84 and 86); lane 25 negative control.



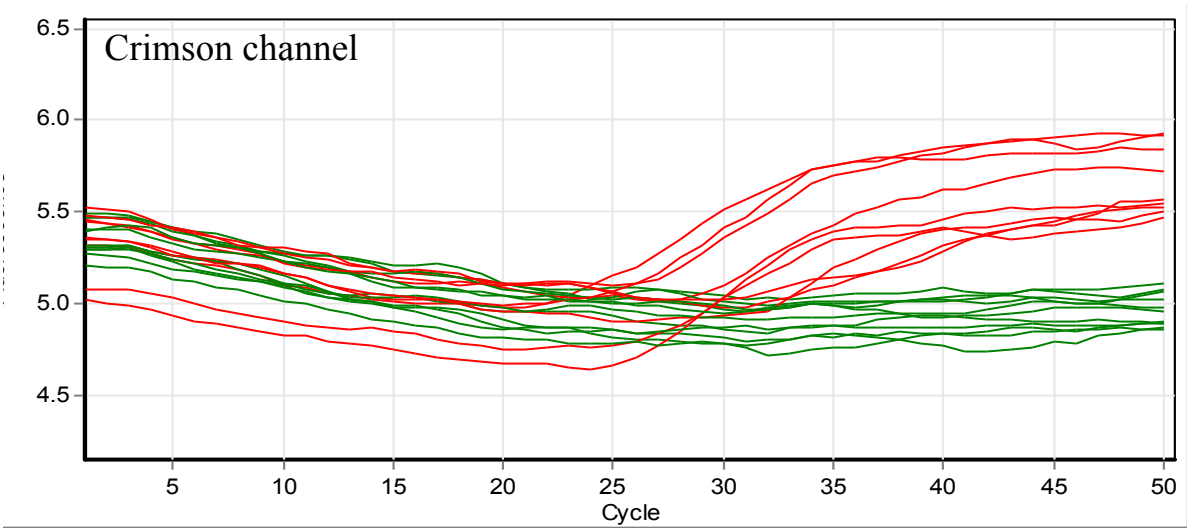
**Figure B.6** Amplification of *cdtA* 221 bp products and *cdtB* 262 bp products by conventional PCR using stool DNA of 24 stool samples. Lanes 1 is DNA molecular weight marker VI (154-2176 bp); Lanes 02-25 representing stool samples number (3, 6, 15, 16, 21, 24, 28, 30, 33, 34, 45, 47, 48, 61, 62, 65, 72, 73, 80, 85, 87, 88, 90 and 91); lane 26 is a negative control.



**Appendix C:** Multiplex real-time PCR using DNA extracted from stool samples by an automated system.

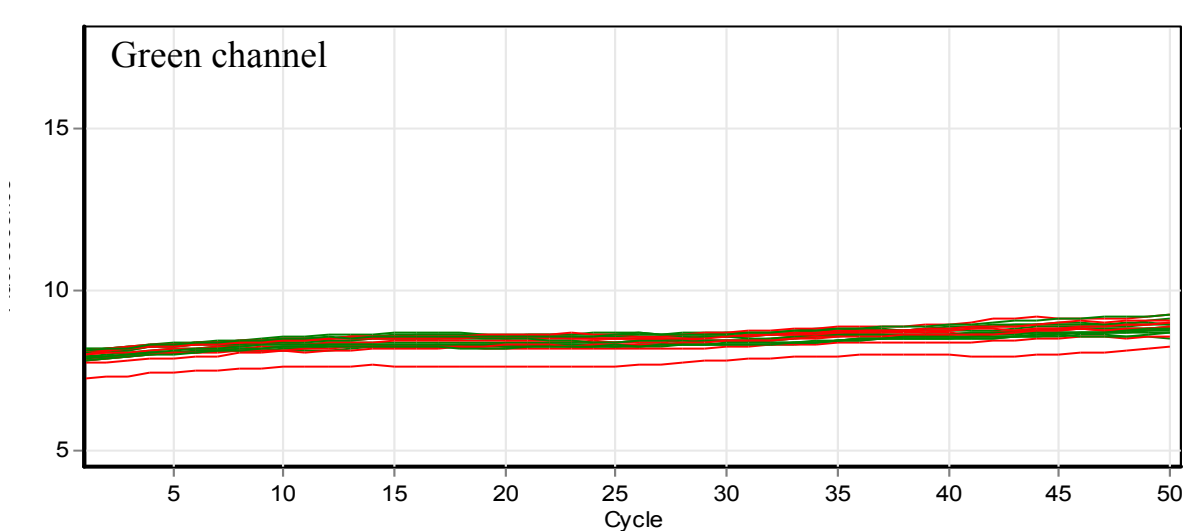


**Figure C.1** Amplification of *tcdA* for samples 46-70 by multiplex real-time PCR. Red curves were *tcdA* positive samples while the green lines were *tcdA* negative samples.

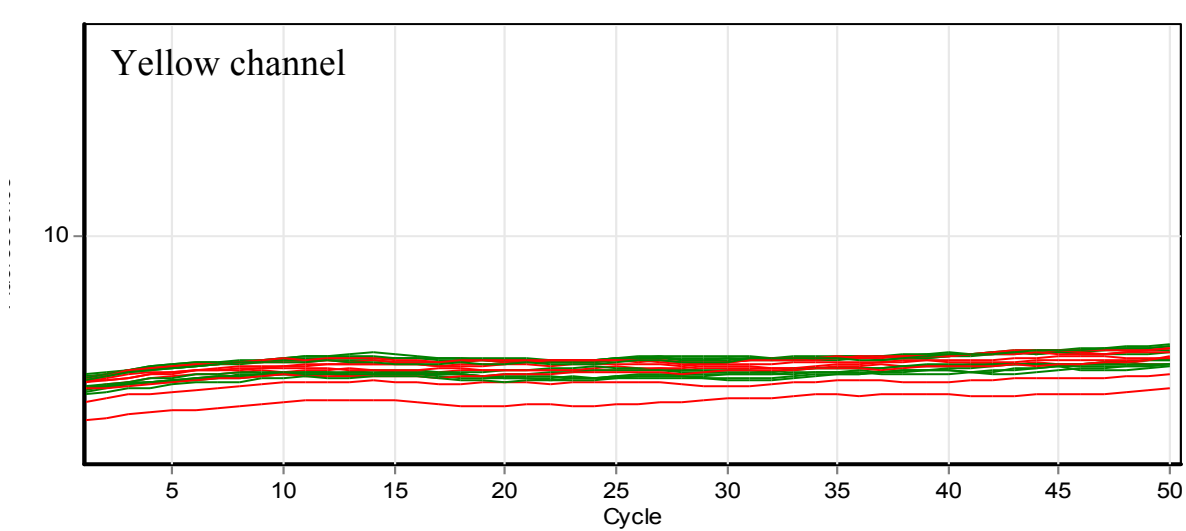


**Figure C.2** Amplification of *tcdB* for samples 46-70 by multiplex real-time PCR. Red curves refer to *tcdB* positive samples while the green lines were *tcdB* negative samples.

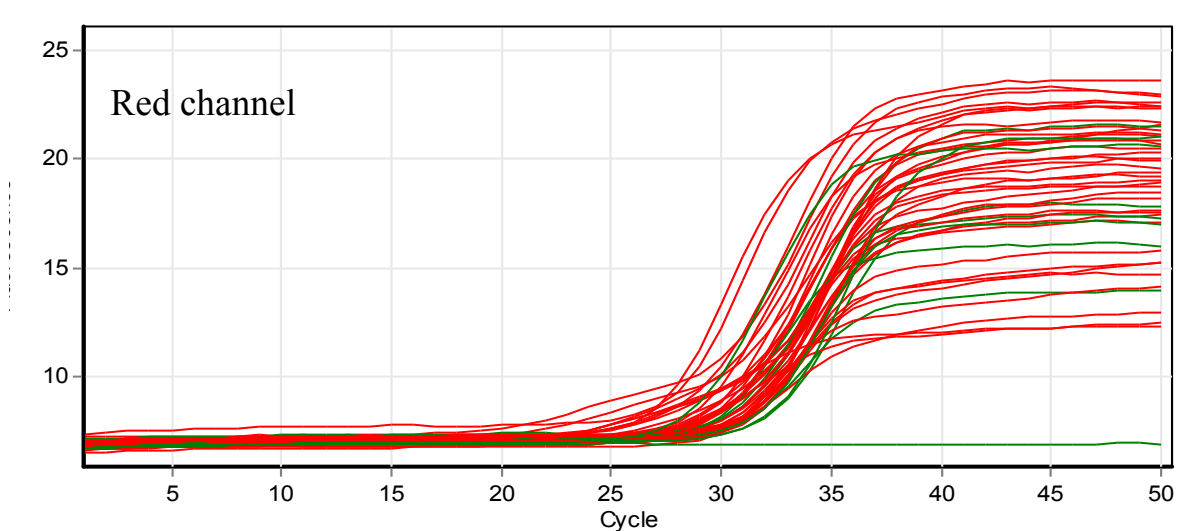




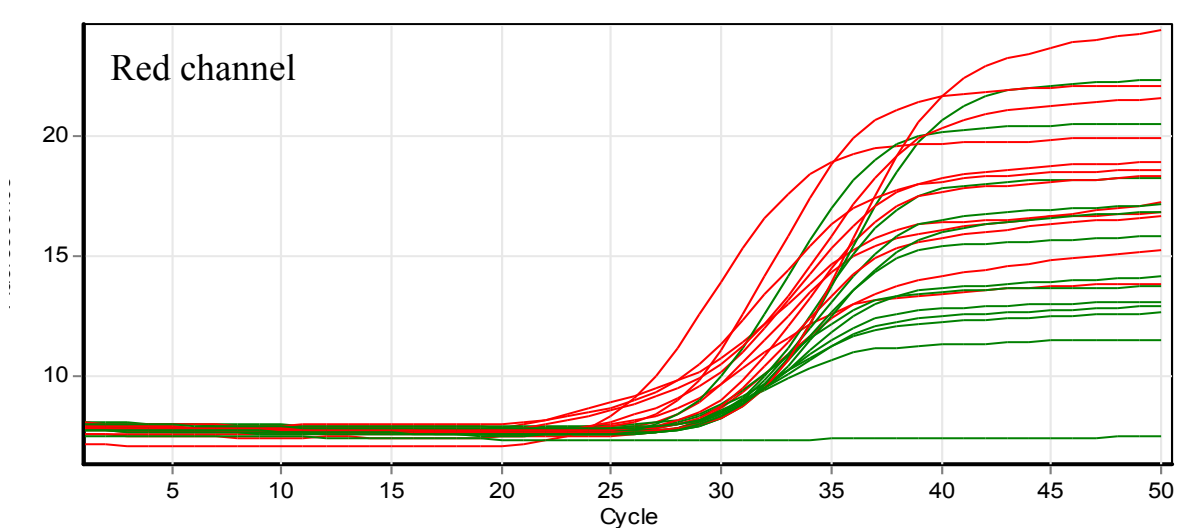
**Figure C.3** Amplification of *cdtA* for samples 46-70 by multiplex real-time PCR. Red and green lines were *cdtA* negative samples.



**Figure C.4** Amplification of *cdtB* for samples 46-70 by multiplex real-time PCR. Red and green lines were *cdtB* negative samples.



**Figure C.5** Amplification of internal control for samples 1-45 by multiplex real-time PCR. Red and green curves were IC positive samples while the green line was NTC.



**Figure C.6** Amplification of internal control for samples 46-70 by multiplex real-time PCR. Red and green curves were IC positive samples while the green line was NTC.